Effects of Sex Steroids on Ovarian Granulosa Cell Function

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To my father,
Ian Wylie Turner

Contents

Declaration

The experiments described in this thesis were the unaided work of the author, except where acknowledgment is made by reference. No part of this work has previously been accepted for any other degree, nor is any part of it being submitted concurrently in candidature for another degree.

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Abstract

The studies described in this thesis were concerned with the role of sex steroids in the control of the differentiation of ovarian granulosa cells. This process is driven by FSH, but is subject to the influence of locally produced factors which are thought to be critical in determining the fate of a follicle. Androgens and oestrogens were known to augment the action of FSH on granulosa cell steroidogenesis, with androgens being the more potent. However, little was known about their mechanism of action, or their effects at the levels of gene expression and protein synthesis. Therefore, a rat granulosa cell culture model was modified and validated to study the role of sex steroids in the control of expression of structurally and functionally important granulosa cell proteins and genes during FSH-stimulated differentiation.

It was found that FSH caused marked changes in the synthesis of a number of prominent cellular and secreted proteins *in vitro*, one of which was identified by immunoprecipitation as vinculin, a cytoskeletal protein, and another as fibronectin, an extracellular matrix protein. Synthesis of both these proteins was reduced in response to FSH. The expression of mRNA encoding another cytoskeletal protein, actin, was also found to be reduced by FSH treatment *in vitro*. All of these effects of FSH, which are consistent with observed changes in morphology during granulosa cell differentiation *in vitro*, were strongly augmented by oestrogen and androgen. However, FSH was found to be without effect on actin mRNA levels *in vivo*. It was concluded that the changes in morphology reflected in these results are probably an artefact of the *in vitro* approach, and so do not provide a suitable model for the study of the role of steroids in ovarian physiology.

A novel aspect of granulosa cell function which was thought to accompany their differentiation was production of inhibin. Little was known about the control of granulosa cell inhibin production, and so the

effects of gonadotrophins and steroids on the expression of mRNA encoding the three inhibin subunits were investigated. FSH stimulated expression of mRNA encoding all three inhibin subunits in rat granulosa cells *in vivo* and *in vitro*. When administered after treatment with FSH, hCG suppressed levels of inhibin subunit mRNA. The relationship between inhibin gene expression and expression of other correlates of granulosa cell differentiation was studied in order to provide a context for these results. Expression of mRNA encoding the LH receptor and the aromatase enzyme was, like the inhibin subunits, increased by FSH and reduced by hCG following FSH treatment, suggesting that all these genes are increasingly expressed during follicle development, and lost at luteinization. The mRNA encoding the side-chain cleavage enzyme, however, was only moderately increased by FSH treatment, but greatly increased by hCG treatment, consistent with its function in the corpus luteum. Since the pattern of expression of all these markers followed qualitatively the same pattern *in vivo* and *in vitro*, the control of inhibin subunit gene expression by FSH and steroids *in vitro* could then be studied in detail. Both androgen and oestrogen augmented the stimulation by FSH of inhibin gene expression in a dose-dependent fashion. Furthermore, it was found that oestrogen, but not androgen, was able to stimulate the expression of the mRNA encoding the inhibin α- and β_B-subunits, in the absence of FSH.

In order that the control of inhibin gene expression could be studied in a quantitative and systematic manner, an attempt was made to develop a sensitive and quantitative solution hybridisation assay for inhibin-α mRNA. Basic developmental work was carried out, but more validation was required before this assay could be used routinely. During the development of this assay, a low molecular weight RNA species was detected which hybridised to the inhibin-α sense probe, suggesting that it may be an endogenous antisense inhibin-α RNA. It was speculated that this could be involved in the regulation of expression of this gene, but further work would be required to determine its function.

The mode of action of androgens and oestrogens in granulosa cells was also investigated by studying the expression of mRNA encoding their receptors. Androgen receptor mRNA was readily detected in granulosa

cells, and its abundance was unaffected by treatment with gonadotrophins. Oestrogen receptor mRNA was also detected in ovarian RNA, but was undetectable in granulosa cells. However, a smaller transcript which hybridised to the oestrogen receptor probe was detected in granulosa cells. The levels of this species were increased by treatment of animals with FSH, and reduced by treatment with hCG following treatment with FSH. Although the function of this molecule is unknown, it is speculated that it may encode an oestrogen-binding protein which may act to regulate levels of free oestrogen in the cell.

It was concluded that oestrogen and androgen interact with FSH to stimulate all aspects of granulosa cell differentiation studied, and that their different potencies can be accounted for in part by different levels of expression of their receptors. However, the ability of oestrogen to stimulate inhibin gene expression in the absence of FSH suggests some difference in their mechanisms of action. Since thecal androgen synthesis is known to be stimulated by inhibin and may be inhibited by oestrogen, it is speculated that this effect of oestrogen may act to maintain supply of androgen substrate for oestrogen synthesis in the presence of high local levels of oestrogen present during preovulatory follicular development.

Abbreviations

2-OH-E_2	2-hydroxyoestradiol (1,3,5[10]-oestratriene-2,3,17β-triol)
3β-HSD	3β-hydroxysteroid dehydrogenase/Δ^{5-4} isomerase
ABP	androgen-binding protein
APS	ammonium persulphate
(d)ATP	(2'-deoxy-) adenosine 5'-triphosphate
bp	base pair(s)
BSA	bovine serum albumin
βME	2-mercaptoethanol (β-mercaptoethanol)
cAMP	cyclic adenosine 3',5'-monophosphate
CG	chorionic gonadotrophin
CRE	cyclic AMP response element
CREB	cyclic AMP response element-binding protein
(d)CTP	(2'-deoxy-) cytidine 5'-triphosphate
DES	diethylstiboestrol
DHT	5α-dihydrotestosterone (5α-androstan-17β-ol-3-one)
(c)DNA	(complementary) deoxyribonucleic acid
DNase	deoxyribonuclease
DTT	dithiothreitol
E_2	oestradiol-17β (1,3,5[10]-oestratriene-3,17β-diol)
EDF	erythroid differentiation factor
EDTA	ethylenediaminetetraacetic acid
EGF	epidermal growth factor
(b)FGF	(basic) fibroblast growth factor
FSH	follicle-stimulating hormone
GDP	guanosine 5'-diphosphate
GH	growth hormone
GnRH	gonadotrophin-releasing hormone
(d)GTP	(2'-deoxy-) guanosine 5'-triphosphate
h	human

HDL	high-density lipoprotein
HEPES	N-(2-hydroxyethyl)piperazine-N'-(2-ethanesulphonic acid)
HMG CoA	3-hydroxy-3-methylglutaryl coenzyme A
HRE	hormone response element
hsp90	90 kilodalton heat-shock protein
ICI 164,275	N-*n*-butyl-11-(1,3,5[10]-oestratriene-3,17β-diol-7β-yl)-undecanamide
ICI 164,384	N-*n*-butyl-11-(1,3,5[10]-oestratriene-3,17β-diol-7α-yl)-N-methylundecanamide
IGF(-I or -II)	insulin-like growth factor (type I or II)
IGF-BP	Insulin-like growth factor-binding protein
IPTG	isopropyl β-D-thiogalactopyranoside
kb	kilobase(s)
K_d	dissociation constant
kDa	kilodalton(s)
KIU	kallikrein inhibitory unit(s)
LB	Luria-Bertani
LDL	low-density lipoprotein
LH	luteinizing hormone
MEM	minimum essential medium
MIS	Mullerian duct-inhibitory substance
MOPS	3-(N-morpholino)propanesulphonic acid
NADH	β-nicotinamide adenine dinucleotide, reduced
NADPH	β-nicotinamide adenine dinucleotide phosphate, reduced
NP-40	Nonidet P-40
(d)NTP	any (deoxy-) nucleotide triphosphate
o	ovine
P450arom	aromatase cytochrome-P450
P450c17	17α-hydroxylase/C17-20 lyase cytochrome-P450
P450scc	side-chain cleavage cytochrome-P450
PAGE	polyacrylamide gel electrophoresis
(D)PBS	(Dulbecco's) phosphate-buffered saline
PCR	polymerase chain reaction

PDGF	platelet-derived growth factor
PMSG	pregnant mare's serum gonadotrophin
prog	progesterone (4-pregnene-3,20-dione)
RIIβ	β-subunit of type II cAMP-dependent protein kinase
RIA	radioimmunoassay
(m or t)RNA	(messenger or transfer) ribonucleic acid
RNase	ribonuclease
SCH 16423	hydroxyflutamide (α,α,α-trifluoro-2-methyl-4'-nitro-*m*-lactotoluidide)
SDS	sodium dodecyl sulphate
SHBG	sex hormone-binding globulin
T	testosterone (4-androsten-17β-ol-3-one)
TCA	trichloroacetic acid
TEMED	N,N,N'N'-tetramethylethylenediamine
TGF(-α or -β)	transforming growth factor (type α or β)
tris	tris(hydroxymethyl)aminomethane
TTP	thymidine 5'-triphosphate
UTP	uridine 5'-triphosphate
UV	ultraviolet
X-Gal	5-bromo-4-chloro-3-indolyl β-D-galactopyranoside

Chapter 1 Literature Review

1 Introduction

In mammals, the ovary has two functions, firstly to release viable gametes at the appropriate time to allow fertilization, and secondly to secrete steroids which influence sexual behaviour around the time of ovulation and prepare the female for pregnancy and lactation. These functions are really inseparable, since steroids not only affect sexual behaviour and organs such as mammary glands and uterus, but also play a pivotal role in the control of ovarian function.

There are two major levels at which control is exerted over the growth and development of ovarian follicles during the normal ovarian cycle. In cyclic animals, the normal development of ovarian follicles, ovulation and luteal function are primarily under the endocrine control of the pituitary gonadotrophins follicle-stimulating hormone (FSH) and luteinizing hormone (LH), and secondarily under paracrine control by factors produced within the ovary. It is of great significance that the ovarian follicle is not simply the passive target organ for pituitary hormones, but is able to modify the production of, and its own response to those hormones through local and endocrine action of steroid and peptide factors. It must be stressed that the use of terms such as "primary" and "secondary" is not meant to indicate greater or lesser importance of one level of control over another, since both mechanisms are necessary for normal follicle development.

This project has focussed on the interactions of steroid and peptide factors in the local control of ovarian function. In this review, I will attempt to provide the background to the ovary and its endocrine control, and the events which occur during the ovarian cycle, before describing in

detail the cell biology of the ovarian follicle and its control at the paracrine level.

2 Structure and Cellular Composition of the Ovary

The mammalian ovaries are paired organs situated in the peritoneal cavity. Their exact position and shape varies between species, but they are generally roughly bean-shaped and approximately equal in size. The ovaries are attached to the broad ligament by a mesentery called the mesovarium, through which the ovarian blood vessels, lymphatics and sympathetic nerves pass before joining the ovary at the ovarian hilus. The ovarian arteries are spiral and form extensive connections with the ovarian veins in the hilar region. The ovary is surrounded by a capsule, or bursa, and the extent to which it encloses the ovary varies between species. In rats and mice, the bursa almost completely encloses the ovary, with very little connection with the peritoneum. In pigs and sheep, the bursa is open to the peritoneum, and in humans it is virtually absent (Harrison, 1962). The ovary itself is covered with a continuous smooth epithelium, called the germinal epithelium. During embryonic development, this epithelium proliferates to give rise to the underlying cortex, in which the germ cells are embedded (Franchi *et al.* 1962). In the cortex of the ovary, a number of cell types and structures can be seen. Apart from the oocytes and the associated cells of the follicle which are described in detail below, the cortical stroma contains connective tissue, fibroblasts, blood and lymphatic vessels and macrophages, as well as secretory cells known as interstitial cells. Primary interstitial cells are steroidogenic cells present only for a short time during fetal life, which are not gonadotrophin-responsive and appear to be involved in the conversion of placental progestogens to androgens (Erickson *et al.* 1985). There are also androgen-producing interstitial cells in the hilar region of the ovary, but their function is unknown. The third type of steroidogenic interstitial cell is the secondary interstitial cell. These cells are thought to be derived from the theca-lutein cells of regressed corpora lutea and are functionally indistinguishable from theca cells (Erickson *et al.* 1985).

During foetal life the germ cells in the ovary proliferate by mitosis to form the oogonia, and enter the first meiotic division. Once meiosis has begun, the germ cells are termed primary oocytes, and they will not proliferate any further. As a result, the number of germ cells in the ovary is fixed by the time of birth, and are not replaced as they are released. Before birth most of these oocytes become surrounded by spindle-shaped cells, which are the precursors for the granulosa cells, to form primordial follicles, which are distributed in the ovarian cortex, just under the germinal epithelium. These granulosa cell precursors form gap junctions with the oocyte. This unit is enclosed from the surrounding stroma by a basement membrane, the membrana propria. With the formation of the primordial follicle, the oocytes arrest in diplotene of the first meiotic prophase, before spindle formation, and at this stage of their growth are unable to proceed any further. Although little is known about the influence of the oocyte on the development of the follicle, it is an essential component since follicles do not form in their absence (Buccione *et al.* 1990).

The majority of the oocytes in the ovary from around the time of birth are present in primordial follicles, but every day of reproductive life, a number of follicles begins to grow. The spindle-shaped cells surrounding the oocyte become cuboidal and begin to proliferate, and are then termed granulosa cells. The oocyte, still arrested in diplotene, approximately doubles in size, and an opaque layer between the granulosa cells and the oocyte, called the zona pellucida is formed from glycoproteins secreted by the oocyte (Philpott *et al.* 1987; Lira *et al.* 1990). The granulosa cells maintain their close contact with the oocyte by forming cytoplasmic projections which extend through the zona pellucida to form gap junctions with the oocyte plasma membrane. The granulosa cells act to promote the growth of the oocyte, by passing nutrients such as nucleotides and lactate to the oocyte (Heller & Schultz, 1980). Granulosa cells function in a specific manner in their effects on oocyte growth, since other cell types are unable to stimulate oocyte growth (Buccione *et al.* 1990). Stromal cells condense around the membrana propria and develop cytoplasmic organelles characteristic of steroid-secretory cells, to form the theca layer. This layer, unlike the

granulosa cell layer, is highly vascular. The follicle is now termed a primary follicle. It is at this stage that the oocyte stops growing, although it continues to produce RNA and protein (Lintern-Moore & Moore, 1979). The growth of the oocyte is associated with development of the ability of the oocyte to resume meiosis, since reinitiation of meiosis occurs spontaneously in oocytes isolated from follicles at this stage (Eppig & Downs, 1984). They do not resume meiosis, however, until just before ovulation, and it is thought that the oocyte is held in this state of meiotic arrest by factors produced by the granulosa cells. Cyclic adenosine 3',5'-monophosphate (cAMP) is able to maintain oocytes in meiotic arrest, and it is thought that granulosa cells, stimulated by FSH, may pass cAMP to the oocyte through gap junctions (Eppig & Downs, 1984). Purine factors such as hypoxanthine, which is present in high concentration in follicular fluid, are also implicated in the maintenance of oocyte meiotic arrest by maintaining high levels of cAMP within the oocyte (Downs & Eppig, 1987; Downs *et al.* 1989).

As the follicle continues to grow, the theca layer differentiates into the theca externa, a fibrous wall to the follicle, and the theca interna, which is a highly vascular steroidogenic layer. The granulosa cells secrete a viscous fluid containing proteoglycans, which eventually coalesces to become the follicular fluid which forms the follicular antrum. At this point, two types of granulosa cell can be distinguished. Those granulosa cells which are in direct contact with the oocye are called the cumulus oophorus, or cumulus granulosa cells, whereas those which lie between the basement membrane and the antrum are known as mural granulosa cells. Up to this stage, follicular growth is relatively independent of gonadotrophins, since follicles will grow to the early antral stage in hypophysectomized animals (Hirshfield, 1985), although this does not mean that these follicles are not gonadotrophin-responsive (Greenwald & Terranova, 1988). In the absence of gonadotrophins, many follicles become atretic, and healthy follicles will proceed no further than the early antral stage.

The follicle is the functional unit of the ovary, with growing follicles functioning independently of each other. The selection of the oocyte or oocytes to be ovulated in a given cycle is exerted on the follicle as a whole.

3 Role of Pituitary Hormones in Follicular Development

The anterior pituitary secretes two gonadotrophic glycoprotein hormones, follicle-stimulating hormone (FSH) and luteinizing hormone (LH). FSH and LH are both heterodimeric glycoproteins, consisting of an α-subunit of 89 amino acids with two glycosylation sites, common to both hormones (and also common to thyroid-stimulating hormone [TSH] and chorionic gonadotrophin [CG]), and a β-subunit 115 of amino acids, which confers specificity. The β-subunits of the two hormones are identical at only seven amino acids, and the FSH β-subunit has two glycosylation sites, whereas the LH β-subunit has only one. The molecular weight of FSH is 32,600 Da, and that of LH is 34,000 Da (Johnson & Everitt, 1988).

3.1 Function of FSH

Follicle-stimulating hormone, as the name suggests, stimulates growth of ovarian follicles *in vivo*. Administration of this hormone *in vivo* leads to marked growth of follicles (Goldenberg *et al.* 1973; Goldenberg *et al.* 1972; Simpson *et al.* 1941; Greep *et al.* 1942), and this has been shown to be due to an increase in the proliferation of the granulosa cells (Roy & Greenwald, 1991; Gougeon & Testart, 1990). The receptor for FSH is present only on granulosa cells of follicles from the large preantral stage onwards, and is not present in the cells of primordial follicles or old corpora lutea (Oxberry & Greenwald, 1982; Camp *et al.* 1991). FSH does not stimulate granulosa cells to grow *in vitro* (Skinner *et al.* 1987; Hammond & English, 1987; Dorrington *et al.* 1988), suggesting that this effect of FSH is mediated through an intermediate *in vivo* (see below). However, through generation of intracellular cAMP (see below), FSH directly causes granulosa cells to differentiate. The major characteristic of this differentiation is the development of steroidogenic activity. FSH stimulates immature granulosa cells, which secrete little or no steroids, to produce progestogens (Hillier *et al.* 1978; Thanki & Channing, 1978; Richards *et al.* 1979; Moon, 1981; Carson *et al.* 1979) and in particular to aromatize androgens to oestrogens (Dorrington *et al.* 1975; Moon *et al.*

1978; Gore Langton & Dorrington, 1981; Hillier & deZwart, 1982). The molecular basis of this aspect of granulosa cell differentiation is discussed below. Another characteristic of granulosa cells which have been induced to differentiate in response to FSH is the expression of receptors for LH (Zeleznik *et al.* 1974; Richards *et al.* 1976; Richards *et al.* 1979; Erickson *et al.* 1979; Sheela Rani *et al.* 1981), which is also linked to the cAMP second messenger system (see below). This is of great importance, since granulosa cells in an advanced stage of differentiation are able to respond to LH in the same way as FSH when levels of FSH are low. FSH also stimulates production of its own receptor (Richards *et al.* 1976; Ireland & Richards, 1978), further adding to the sensitivity of these cells to gonadotrophin stimulation. Granulosa cells also produce a number of important proteins in response to FSH, including proteoglycans (Ax & Ryan, 1979; Yanagishita *et al.* 1981), which contribute to the formation of follicular fluid; inhibin and follistatin (Bicsak *et al.* 1986; Michel *et al.* 1991b; Hillier *et al.* 1989b; Michel *et al.* 1990), which are probably involved in the control of FSH secretion (see below) and may also be local regulators of follicular function (see below); plasminogen activators (Canipari & Strickland, 1986; Ny *et al.* 1987; O'Connell *et al.* 1987; Galway *et al.* 1990), which are important in ovulation (see below); and a variety of growth factors and their receptors (Adashi *et al.* 1985a; Hsueh, 1986; Hillier, 1990; Adashi *et al.* 1991), which may be involved in the control of follicle development and tissue remodelling during the major changes in structure of the follicle during the ovarian cycle (see below).

Differentiation of granulosa cells *in vitro* is also accompanied by profound morphological changes. Upon stimulation with FSH, cultured granulosa cells become rounded and aggregate (Soto *et al.* 1986), due to cytoskeletal reorganization. Stress fibres are dismantled (Amsterdam & Rotmensch, 1987), and production of the major components of stress fibres, actin and α-actinin, falls (Ben Ze'ev & Amsterdam, 1987; Amsterdam & Rotmensch, 1987; Anderson *et al.* 1988). Production of fibronectin (Skinner *et al.* 1985; Skinner & Dorrington, 1984), which is an extracellular matrix protein, and vinculin, which is involved in linking stress fibres to fibronectin, is also suppressed during FSH-induced differentiation (Ben Ze'ev & Amsterdam, 1986; Amsterdam &

Rotmensch, 1987; Ben Ze'ev & Amsterdam, 1987). FSH also stimulates the formation of gap junctions between granulosa cells (Lindner *et al.* 1977; Ben Ze'ev & Amsterdam, 1987), allowing them to pass nutrients between each other and to the oocyte, which becomes increasingly important as the follicle grows, since the granulosa cell layer has no direct blood supply. The formation of gap junctions between granulosa cells allows other molecules to be passed throughout the granulosa layer. In particular, cAMP produced in response to gonadotrophins can be passed through gap junctions, so that cells devoid of gonadotrophin receptors can still respond through coupling with their neighbours (Lawrence *et al.* 1978).

Therefore, FSH acts on granulosa cells not only to drive the growth of the follicle and production of steroid and protein hormones, but also to increase the sensitivity of the granulosa cells to gonadotrophins through increased expression of their receptors, functional coupling via gap junctions as well as through production of steroids and protein factors which, as discussed below, also act to increase the sensitivity of the follicle to gonadotrophins.

3.2 Function of LH

LH administered *in vivo* in the absence of FSH leads to the growth of follicles (Simpson *et al.* 1941; Greep *et al.* 1942). This effect is not so marked as the effect of FSH, and is largely due to thecal hypertrophy (Erickson *et al.* 1985). When administered in conjunction with FSH, however, LH triggers ovulation and subsequent formation and maintenance of corpora lutea (Greep *et al.* 1942; Lostroh & Johnson, 1966; Smith & Bradbury, 1966), hence its name.

Until late follicular development, receptors for LH are limited to the theca compartment of the follicle, where LH acts to stimulate the production of a number of factors, in particular androgens and progestogens (Erickson *et al.* 1985; Erickson & Ryan, 1976; McNatty *et al.* 1980; Tsang *et al.* 1979b). LH therefore acts in the developing follicle to stimulate the theca cells to provide the granulosa cells with androgen substrate for the synthesis of oestrogens. As the follicle grows, its

granulosa cells mature in response to FSH, and express receptors for LH. Such granulosa cells respond to low levels of LH by producing oestrogen, progesterone, inhibin and other factors, in the same way as they do to FSH (Hillier, 1985; Hsueh *et al.* 1984). High levels of LH cause ovulation and luteinisation of granulosa cells. These granulosa-lutein cells then secrete progesterone in response to LH (Moor, 1974).

3.3 Signal Transduction

Gonadotrophins bind to specific membrane receptors, which are members of a large family of structurally related membrane receptors. Members of this family are large, single chain polypeptides with seven regions of hydrophobic residues, separated by short stretches of hydrophilic amino acids. The hydrophobic regions of the receptors span the cell membrane, and the hydrophilic regions connect these membrane-spanning regions, forming intracellular and extracellular loops (Johnson & Dhanasekaran, 1989). The hormone recognition site of the receptor resides in the long extracellular amino terminus. The LH/hCG receptor has been studied in some detail, and its cDNA recently cloned (Bernard *et al.* 1990; Minegishi *et al.* 1990; McFarland *et al.* 1989). The molecular weight of the ovarian LH receptor has been reported to be between 85kDa and 93kDa in its monomeric glycosylated form (Minegishi *et al.* 1989; Rosemblit *et al.* 1988) and between 59kDa and 62kDa when deglycosylated (Minegishi *et al.* 1989; Petaja Repo *et al.* 1991). The glycosylation of the hormone-binding domain may be involved in the interaction of the receptor with the ligand(Dufau *et al.* 1989; Minegishi *et al.* 1989), although this has recently been disputed (Petaja Repo *et al.* 1991). Certainly, the glycosylation of the ligand (LH or hCG) is important to its binding (Petaja Repo *et al.* 1991; Sairam *et al.* 1990). It has also been reported that the receptor exists in the membrane as a dimer (Dufau *et al.* 1989; Zhang & Menon, 1989). As well as activation of adenylate cyclase, binding of ligand to the LH receptor leads to phosphorylation by protein kinase A (Dufau *et al.* 1989; Minegishi *et al.* 1989), and this may be involved in the striking down-regulation of the receptor by LH (Rosemblit *et al.* 1988). Less is known about the FSH receptor, but since it

has recently been cloned, no doubt more information will soon be available. Based on its cDNA sequence, the FSH receptor has a predicted deglycosylated molecular weight of approximately 75kDa, with a long (348 amino acids) extracellular hormone-binding domain which is potentially glycosylated in three positions (Sprengel *et al.* 1990). The FSH receptor bears high sequence homology with the LH/hCG receptor, as would be expected from the similarity of the ligands, especially in the transmembrane regions.

Both receptors are functionally coupled to G-proteins (Reichert & Dattatreyamurty, 1989), and the intracellular loops are thought to be the site of this interaction (Johnson & Dhanasekaran, 1989). G-proteins consist of three subunits, designated α, β and γ in descending order of molecular weight. It is the α-subunit which appears functionally to be the most important component of the G-proteins, although little is known about the functions of the other subunits other than that they serve to anchor the inactivated α-subunit to the cell membrane. The α-subunit contains a region in its carboxy terminus which binds to a part of the receptor to which it is coupled, probably by one of the intracellular loops of the receptor (Johnson & Dhanasekaran, 1989). Antibodies to this region of the G-protein α-subunit are capable of uncoupling the G-protein from its receptor (Spiegel *et al.* 1990; Milligan *et al.* 1990). Another region of the α-subunit binds GDP in its inactivated state, but upon agonist binding to the receptor, the receptor catalyses the exchange of bound GDP for GTP, the predominant form of guanosine phosphate in the cell, is bound instead. This results in dissociation of the α-subunit from the other subunits, and in this free state the α-subunit exerts its action on intracellular second messenger metabolism. The α-subunit possesses intrinsic GTPase activity, and converts its bound GTP to GDP, returning the protein to its inactive membrane-bound form (Johnson & Dhanasekaran, 1989).

G-protein α-subunits exist in a number of forms which stimulate or inhibit production of intracellular second messengers or ion channel opening, depending on their type. The type of G-protein α-subunit coupled to gonadotrophin receptors is a stimulatory (denoted α_s) protein, which activates the membrane-bound enzyme adenylate cyclase (Spiegel *et al.* 1988). This enzyme converts adenosine diphosphate (ADP) to cAMP,

which acts as the second messenger mediating the actions of both FSH and LH. cAMP exerts its effects through activation of cAMP-dependent protein kinases (protein kinase A). Protein kinase A consists of four subunits; two regulatory cAMP-binding subunits, and two catalytic subunits (Taylor *et al.* 1990). Binding of cAMP to the regulatory subunits allows phosphorylation of a variety of proteins, resulting in acute effects, for example on steroidogenesis, and also longer term effects on gene expression. The exact mechanism whereby activation of protein kinase A leads to changes in gene expression is not fully understood. A transcription factor known as cAMP response element-binding protein (CREB) has been identified, which binds as a dimer to an enhancer element known to mediate cAMP responsive gene expression, the cAMP response element (CRE), and stimulates transcription from genes under the regulation of a CRE (Montminy *et al.* 1990). It is likely that protein kinase A directly or indirectly activates CREB, leading to expression of gonadotrophin-responsive genes in ovarian cells.

LH, presumably through LH receptor activation of a different G-protein intermediate, is also capable of stimulating release of inositol phosphates and diacylglycerol from thecal and granulosa cell membranes, and is therefore also able to activate protein kinase-C (Davis *et al.* 1983; Davis *et al.* 1984; Davis *et al.* 1986a; Dimino *et al.* 1987). The contribution of this second messenger pathway in the mediation of LH effects is not clear.

3.4 Control of Gonadotrophin Secretion

FSH and LH are released in response to stimulation of the gonadotrophs by the hypothalamic decapeptide, gonadotrophin-releasing hormone (GnRH). This factor is synthesized in the cell bodies of neurones situated mainly in the medial preoptic area and the arcuate and paraventricular nuclei of the hypothalamus, and transported to their terminals in the median eminence. GnRH is then released into the hypophysial portal vein in a pulsatile fashion and delivered to the anterior pituitary by the blood (Johnson & Everitt, 1988). The nature of the receptor for GnRH is still obscure, but it is likely to be another

member of the seven transmembrane domain G-protein coupled receptor family. GnRH acts to stimulate release of gonadotrophins acutely, and also to stimulate expression of the genes encoding gonadotrophin β-subunits. GnRH also acts to increase numbers of its own receptor (the so-called self-priming effect). These effects are mediated through a calcium-dependent mechanism, probably through activation of protein kinase C (Conn, 1989).

LH is released from intracellular stores in pulses in response to pulses of GnRH, whereas FSH release is more dependent upon its rate of synthesis, and is therefore released more or less continuously (McNeilly, 1988).

The secretion of gonadotrophins is under control at the level of both pituitary and hypothalamus. Circulating steroid and protein factors produced by the ovary exert both negative (especially on FSH) and positive feedback on the secretion of gonadotrophins. The major steroid involved in the control of FSH secretion is oestradiol, although high concentrations of progesterone exert similar suppressive effects at the pituitary. At low circulating levels of oestradiol, FSH secretion is rapidly inhibited. This action of oestradiol is mainly exerted at the pituitary, where it acts to inhibit transcription of both the α-subunit and particularly the β-subunit of FSH (Phillips *et al.* 1988). The suppression of secretion of FSH by oestradiol or progesterone is very rapid and profound, and can be accounted for entirely by this suppression of FSH β-subunit mRNA synthesis. Although steroids inhibit transcription of the α-subunit, LH levels remain fairly constant while FSH levels fall (Baird, 1991), because secretion of FSH is more dependent upon its rate of synthesis than LH (see above).

Although low concentrations of oestradiol acutely suppress FSH secretion, longer exposure to high concentrations results in a massive release of LH, and to a lesser extent FSH. It is this surge of LH which triggers ovulation. The mechanism of oestrogen positive feedback involves action both at the pituitary, where it progressively enhances sensitivity to GnRH over the late follicular phase (Johnson & Everitt, 1988) due to increased GnRH receptor numbers (Laws *et al.* 1990), and the hypothalamus, where it increases GnRH pulse frequency at the time of

the LH surge, probably by an indirect action on non-GnRH-secreting neurones (Fink, 1988). Progesterone is able to prevent the LH surge in response to oestradiol (Johnson & Everitt, 1988), partly by its negative effects on FSH synthesis at the pituitary, and partly by slowing GnRH pulse frequency at the hypothalamus (Ramirez *et al.* 1985), explaining why the large amounts of oestradiol secreted throughout the primate luteal phase fail to elicit a surge of LH.

A number of proteins potentially produced in the ovary may also be involved in the control of FSH secretion. Inhibin (see below) has been shown in a number of species to suppress FSH synthesis and secretion by cultured pituitary cells (Robertson *et al.* 1986; Farnworth *et al.* 1988), and immunoneutralization of inhibin *in vivo* results in an increase in plasma FSH levels and increased ovulation rate in rats, pigs and sheep (Mann *et al.* 1989a; Rivier & Vale, 1989; Findlay *et al.* 1989; Wrathall *et al.* 1990; Brown *et al.* 1990). Inhibin appears to exert this effect by reducing the increase in FSH β-subunit mRNA in response to GnRH (Carroll *et al.* 1989), probably by blocking the ability of GnRH to up-regulate its own receptor (Braden *et al.* 1990; Busbridge *et al.* 1990; Wang *et al.* 1989c). However, a direct cause-effect relationship between secretion of inhibin by the ovary and falling plasma FSH levels has not been shown. It may be that inhibin does inhibit FSH release *in vivo*, but in humans and sheep at least, it is probably not responsible for the decline in plasma FSH seen in the mid-late follicular phase (Baird, 1991). There is less evidence for the involvement of activin in the control of FSH secretion. This protein has been shown to stimulate FSH secretion by cultured pituitary cells (Schwall *et al.* 1988; Mason *et al.* 1989), by increasing levels and stability of FSH-β mRNA (Attardi & Miklos, 1990; Carroll *et al.* 1991), but to date there is no evidence for the secretion of this protein into the blood. The diverse sites of production of activin, and the discovery that follistatin, another gonadal protein capable of suppressing pituitary FSH secretion (Ying *et al.* 1987; DePaolo *et al.* 1991), is a binding protein for activin (Nakamura *et al.* 1990), seem to argue against an endocrine role for ovarian activin in the control of FSH secretion. One very recent report has shown that injection of activin causes an increase in plasma FSH

levels in rats (Rivier & Vale, 1991), so the question of the systemic role of activin in the control of FSH secretion remains open.

4 The ovarian cycle

The mammalian ovarian cycle consists of a repeated process of follicle recruitment, selection, ovulation and corpus luteum formation and regression. The human menstrual cycle can be split into two functional phases, follicular and luteal. The follicular phase is the part of the cycle during which small follicles are stimulated to grow in response to an initial rise in FSH levels and tonic LH levels. As this phase progresses, the appropriate number of follicles which begin to secrete oestrogen are "selected", while others become atretic. During this phase tonic LH levels are maintained and FSH levels gradually fall as oestrogen secretion by the dominant follicle(s) rises. The transition from the follicular phase to the luteal phase is marked by a surge of gonadotrophin from the pituitary, followed some hours later by ovulation. The ovulated follicle becomes the corpus luteum, which in humans secretes progesterone, oestradiol and inhibin. The lifespan of this gland in a non-fertile cycle is the length of the ensuing luteal phase. After regression of the corpus luteum, gonadotrophin levels rise again, triggering another cycle. The events of the cycle are governed by the secretion of gonadotrophins by the pituitary, and the resulting secretion of steroids and other factors, which exert feedback control over gonadotrophin secretion. Hence, the cyclicity of the process is an intrinsic property of the interaction between the ovary and the pituitary (and hypothalamus), and in humans and other non-seasonal species is not imposed by the central nervous system.

The relative durations of the two phases of the cycle and the number of eggs ovulated in each cycle vary between species. Species can be classified very roughly into three groups on the basis of the relative lengths of the follicular and luteal phases of their cycles. The first group of species, which includes humans and most other primates, have follicular and luteal phases of approximately the same length. Development of small follicles does not begin in these species until the

corpus luteum regresses. This may be because the corpus luteum in these species secretes oestrogen and inhibin as well as progesterone (Baird, 1991; Bassett *et al.* 1990), resulting in suppression of gonadotrophin secretion from the pituitary. As a result, the follicle must go through the whole process of gonadotrophin-dependent development after regression of the corpus luteum.

Some other species, such as pigs, guinea pigs, cows and sheep, have a luteal phase roughly equivalent to that of the human, but their follicular phase is much shorter. This is probably because their corpora lutea secrete no oestrogen or inhibin (Greenwald, 1979; Mann *et al.* 1989b; Henderson & Franchimont, 1983). Consequently, levels of gonadotrophin (in particular FSH) are much higher during the luteal phase in these species. This allows follicle development to occur while the corpus luteum is still present.

The third group of mammals which includes rats, mice and hamsters, corpora lutea are not functional (i.e. secrete little progesterone) and will regress in 2-3 days, unless coitus has taken place. Follicular development is triggered in rats by a secondary surge of FSH occurring on the morning of oestrus, at about the same time as ovulation (Barraclough & Wise, 1979). As a result, rats have very short oestrous cycles of four to five days. A functional luteal phase of a length comparable to that of long-cycle species can be induced by mating a female of one of these species with an infertile male. This induced luteal phase is known as pseudopregnancy. In rabbits and cats, even ovulation does not take place without physical stimulation of the cervix, and these species appear to have permanent follicular phases, with repeated waves of antral follicular development and atresia occurring until mating, which triggers an LH surge, leading to ovulation followed by a luteal phase.

The difference in the relative lengths of follicular and luteal phases between primates and other mammals is probably related to their different types of ovarian cycle. Primates such as humans have menstrual cycles in which the endometrium is shed at the end of the luteal phase of every infertile cycle. Consequently, the endometrium must be fully regenerated by the time an embryo reaches the uterus to implant, placing a minimum limit on the interval between ovulations. This may be the

reason for extending the follicular phase by suppression of gonadotrophin secretion during the luteal phase (Baird, 1991).

4.1 Recruitment of Secondary Follicles

As described above, there is a steady trickle of follicles beginning to grow in response to unknown cues and independently of gonadotrophins throughout life. It has been shown that pregnant mare serum gonadotrophin (PMSG), which has both FSH and LH activity, has no effect on the growth of follicles until they have developed a distinct theca layer (Goldenberg *et al.* 1973). By the time these follicles begin to develop an antrum, their granulosa cells have developed receptors for FSH, and their theca cells have developed LH receptors (Oxberry & Greenwald, 1982; Uilenbroek & Richards, 1979). Shortly after the LH surge, FSH levels peak for a second time in many species including the rat and sheep (McNeilly, 1988; Butcher *et al.* 1974; Pant *et al.* 1977). This is thought to be due to a drop in secretion of oestradiol and inhibin by the preovulatory follicle which occurs after the LH surge in these species (Watanabe *et al.* 1990; Campbell *et al.* 1990; Barraclough & Wise, 1979). Since these factors inhibit FSH secretion, it is freed from negative feedback soon after the LH surge. This rise in FSH levels is responsible for the recruitment of the small antral follicles present at that time. In most species, therefore, the development of follicles in a cycle begins at the beginning of the luteal phase of the previous cycle. In primates, the situation is rather different. Because the primate corpus luteum secretes oestradiol and inhibin as well as progesterone, there is no relaxation of negative feedback on the pituitary until regression of the corpus luteum. At this point, FSH levels do rise, and the next wave of small follicles is recruited.

Theca and particularly granulosa cells of these follicles proliferate, and the theca begin to synthesize androgens in response to tonic levels of LH (see below). In the mid to late follicular phase, however, most of these developing follicles will degenerate, or become atretic. A number of follicles, depending on the species, do not become atretic, and are destined to ovulate.

As the recruited follicle increases in size, the granulosa cells within a follicle are not all at exactly the same stage of differentiation. The expression of steroidogenic enzymes is greatest in the granulosa cells closest to the basement membrane (Goldschmit *et al.* 1989; Zlotkin *et al.* 1986; Tabarowski & Szoltys, 1987; Rune & Heger, 1987). The expression of LH receptors follows the same pattern (Oxberry & Greenwald, 1982; Richards *et al.* 1987); the binding capacity of the granulosa cells forming the wall of the follicle is tenfold greater than that of the cumulus cells (Lawrence *et al.* 1980). Conversely, proliferation of granulosa cells as determined by tritiated thymidine uptake is greatest in the cells lining the antrum and surrounding the oocyte (Hirshfield, 1986). Interestingly, expression of mRNA encoding IGF-I, which is implicated in the control of granulosa cell proliferation (see below), is also greatest in the cells lining the antrum (Oliver *et al.* 1989). Despite the presence of gap junctions coupling cells in the granulosa layer, therefore, it does not function entirely as a syncitium. There appears to be a gradient of differentiation, at its highest near the thecal layer and the blood supply, and a gradient of proliferation in the opposite direction. It is likely that this is due to better access in the region nearest the basement membrane to FSH and other factors from the blood, and to regulatory factors such as androgen from the theca. Although cAMP produced by FSH-stimulated (or LH-stimulated) cells can be passed between cells through gap junctions, it appears that it does not penetrate the most distant cells, probably gradually being broken down by phosphodiesterases *en route.*

4.2 Selection of Dominant Follicles

As the follicle develops, the steroidogenic capacity of its granulosa cells increases in response to FSH. Secretion of inhibin by granulosa cells is also stimulated by FSH. As a result, towards the end of the follicular phase of the cycle, concentrations of oestradiol and inhibin in the blood increase which feed back to the pituitary, to suppress the secretion of FSH. Therefore, at this stage of the cycle, large numbers of growing follicles are exposed to declining trophic support. Many of these follicles are unable to withstand this, and become atretic. However, those follicles which are

most advanced are able to continue growing despite lower FSH exposure. Such follicles are termed dominant, and the number of follicles selected in a cycle varies with different species. There is strong evidence that a key factor in the selection of a follicle is its capacity for synthesizing oestrogen.

This ability of a follicle to continue developing despite falling FSH levels is thought to be due to a reduced threshold concentration of FSH required by that follicle for continued growth (Baird, 1987). Since all follicles are exposed to the same concentration of FSH from the blood, it seems likely that this increased sensitivity to FSH is due to the influence of locally produced substances. The relative importance of a number of substances which have been implicated in this mechanism is discussed in detail below. In addition, one of the most important effects of FSH is the induction during the second half of the follicular phase of receptors for LH on the granulosa cell membranes. This receptor, like the FSH receptor, is also coupled to the cAMP signalling system. Therefore, in follicles containing mature granulosa cells possessing LH receptors, tonic levels of LH are able to make up for falling FSH levels.

4.3 Ovulation

As the dominant follicle increases in size, its capacity for oestrogen secretion increases, and levels of oestradiol in the blood increase to a level at which its effects on the pituitary are positive. This leads to a massive release of both FSH and, more importantly, LH. This surge of LH stimulates production of progesterone (Moor, 1974) and prostaglandins (Morioka *et al.* 1989; Wong *et al.* 1989) by the granulosa cells of the preovulatory follicle, both of which are involved in the processes which lead to ovulation. Progesterone promotes ovulation in perifused ovaries (Baranczuk & Fainstat, 1976; Greenwald, 1977; Tsafriri *et al.* 1987), and it has been suggested that it does so by interfering with collagen synthesis in the follicle wall (Tjugum *et al.* 1984). Prostaglandins have also been shown to induce ovulation (Strickland & Beers, 1976), and indomethacin, an inhibitor of prostaglandin synthesis, is able to block this effect (Canipari & Strickland, 1986). The action of prostaglandins may be exerted at several levels. Prostaglandins produced in response to the

gonadotrophin surge lead to increased blood flow to the follicle, accompanied by increased vascular permeability (Brannstrom & Janson, 1991) leading to increased follicular fluid volume. The last phase of preovulatory follicle growth is not due to cell proliferation, but increased follicular fluid volume (Brannstrom & Janson, 1991). Prostaglandins also affect contractility of smooth muscle in the theca externa layer (Morioka *et al.* 1989), although the involvement of muscle contractility in ovulation is uncertain (Brannstrom & Janson, 1991). It is clear, however, that the production of proteases in the follicle is involved in follicular rupture. The major proteolytic enzymes produced by granulosa and theca cells, as well as oocytes, are plasminogen activators (Curry *et al.* 1989; Cajander, 1989). Production of plasminogen activators (especially tissue-type plasminogen activator) is stimulated by both gonadotrophins (Galway *et al.* 1990; Liu *et al.* 1987a; Canipari & Strickland, 1986), and is maximal at around the time of ovulation (Canipari & Strickland, 1985) and the enzyme is most abundant in the apical region of the follicle, where the stigma forms (Liu *et al.* 1987a). Expression of collagenases is also stimulated by human chorionic gonadotrophin (hCG) (Reich *et al.* 1991). These enzymes are believed to play an important part in follicle rupture by degrading connective tissue in the follicle wall, a hypothesis which is supported by the observation that administration of antibodies to plasminogen activator partially blocks ovulation (Tsafriri *et al.* 1989a). Prostaglandins also stimulate granulosa cell plasminogen activator synthesis (Canipari & Strickland, 1986), and it has been suggested that the gonadotrophin stimulation of plasminogen production is actually mediated through prostaglandin synthesis and autocrine action (Canipari & Strickland, 1986). Progesterone is also involved in the synthesis of plasminogen activator, by augmenting the effects of gonadotrophins (Ny *et al.* 1985).

Just prior to ovulation, gap junctions between the cumulus cells and between the oocyte and the cumulus cells break down (Lindner *et al.* 1977), and the cumulus mass expands, due to intercellular accumulation of hyaluronic acid (Eppig, 1991). This may allow the cumulus-oocyte complex to float freely in the antrum, allowing its expulsion when the follicle ruptures. This breakdown of communication between the oocyte

and the granulosa cells and between the cumulus cells and the mural granulosa cells is followed by reinitiation of meiosis by the oocyte, probably due to the removal of inhibitory factors such as cAMP or purines produced by the granulosa cells (see above), or possibly due to removal of electrical coupling of the oocyte and granulosa cells (Lindner *et al.* 1977).

4.4 The Corpus Luteum

Following ovulation, the follicle collapses, the basement membrane fragments (Yoshinaga Hirabayashi *et al.* 1990) and the cells of the follicle wall fill the antrum, to form the corpus luteum. To support the cells in this more solid structure, blood vessels invade the cell mass. The corpus luteum secretes progesterone, and in primates, oestradiol and inhibin as well (Savard *et al.* 1965; McLachlan *et al.* 1987; Fraser *et al.* 1989). The function of this is to provide a secretory endometrium to support pregnancy in the event of conception and implantation, until the placenta has developed sufficiently to take over. Accordingly, the granulosa cells of the former follicle enlarge and accumulate cytoplasmic lipid droplets and large numbers of mitochondria, which are the characteristics of active steroid-secretory cells. This is the final stage of the differentiation of the granulosa cells, and is known as luteinisation. The granulosa-lutein cells lose their responsiveness to FSH, due to a loss of FSH receptors (Lee & Takahashi, 1977; Oxberry & Greenwald, 1982; Richards *et al.* 1976; Camp *et al.* 1991), but remain responsive to LH. The theca-lutein cells also secrete progesterone in response to LH. In some species such as rats and mice, another pituitary hormone, prolactin, also stimulates luteal steroidogenesis (Hilliard, 1973), but its luteotrophic action is less clear in other species. The steroidogenic capacity of the luteal cells is at its greatest at the beginning of the luteal phase and declines thereafter, although their LH responsiveness may increase (Fisch *et al.* 1989). Progesterone concentrations continue to rise during the luteal phase however, and this discrepancy has been explained by the increasing vascularisation of the corpus luteum during the first half of the luteal phase (Niswender *et al.* 1976). During the second half of the luteal phase

of an infertile cycle, progesterone secretion by the corpus luteum falls until the gland regresses altogether. The longevity of the corpus luteum depends upon the species, from a luteal phase accounting for most of the cycle in sheep and pigs, to no true luteal phase at all, unless coitus has taken place, in the rat (Barraclough & Wise, 1979). However, in a particular species, the lifespan of the corpus luteum is fixed, and in an infertile cycle the luteal cells die after that time has elapsed. The regression of the corpus luteum appears not to be dependent upon LH levels, but rather it seems to be an inherent property of the corpus luteum which has been suggested to be a form of "programmed" cell death (Zeleznik, 1991; Hurwitz & Adashi, 1992; Kaynard *et al.* 1992). If pregnancy is established, however, chorionic gonadotrophin (CG) is secreted by the placenta of some species such as the human. This hormone acts on the same receptor as LH, and is able to "rescue" the corpus luteum and prolong its lifespan. Why CG is able to rescue the corpus luteum when LH is not is not yet clear, but is probably related to the greater half-life of CG (Rizkallah *et al.* 1969), and the slower internalisation of CG-receptor complexes by luteal cells (Mock & Niswender, 1983).

5 Ovarian steroidogenesis

Steroid hormones are produced from cholesterol by the action of a variety of separate enzymes, each catalyzing specific reactions. Three classes of steroid hormone are produced in the ovary; progestogens, androgens and oestrogens. Pathways of steroidogenesis in the ovary are summarised in Fig.1.1. The specific steroids produced and how much is produced depends not only on the enzymes present, but also on the availability of substrates. The enzymes are expressed in a cell-specific manner, and their expression is under the direct influence of gonadotrophins and locally produced paracrine factors.

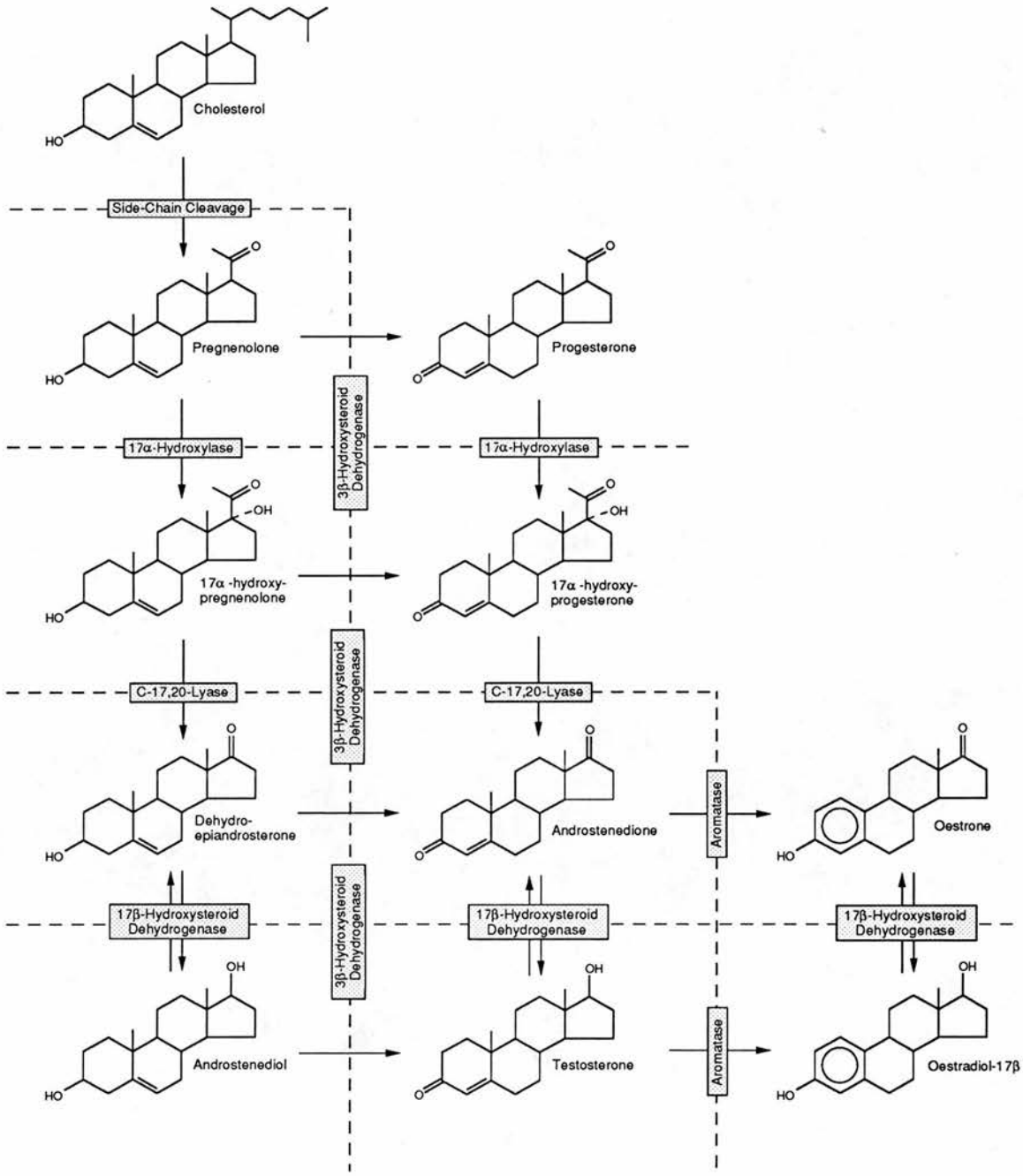


Fig.1.1. Pathways of steroid synthesis in the ovary.

5.1 The Biochemistry of Steroidogenesis

5.1.1 Uptake of Cholesterol

Cholesterol is the compound from which all other steroids are synthesized. Steroidogenic cells are capable of synthesizing cholesterol from acetyl coenzyme A, via a pathway in which the rate-limiting step is catalyzed by 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG CoA reductase). However, given an adequate supply, the preferred source of cholesterol is in the form of blood-borne lipoproteins. Cholesterol is delivered to the ovary in the blood either as low density lipoproteins (LDL) or high density lipoproteins (HDL). The relative amounts of LDL and HDL used as sources of cholesterol varies between species; in humans LDL is the preferred source, whereas in rats cholesterol is mainly taken up from HDL (Miller, 1988; Gwynne & Strauss, 1982).

LDL is taken up by receptor-mediated endocytosis at the microvilli of steroidogenic cells, and the lipoprotein degraded in lysosomes. LDL receptors are recycled to the plasma membrane. The mechanism by which cholesterol is taken up from HDL is less clear. It is thought that the HDL associates with a recognition site on the plasma membrane (Ghosh & Menon, 1986), but rather than being internalized, the HDL may release its cholesterol, through the action of lipases present on the surface of the cell, which then passes into the cell.

Cholesterol is stored within the cell in lipid droplets, esterified to fatty acids. The synthesis of cholesterol esters is catalyzed by acyl coenzyme A: cholesterol acyltransferase (ACAT), and their breakdown, to yield free cholesterol for metabolism to steroids, is catalyzed by cholesterol esterase. During active steroidogenesis, cholesterol is mobilized from stored cholesterol esters, and transported to the mitochondria, where the first stage of steroidogenesis takes place. The transport of cholesterol to the mitochondria remains obscure, but it probably involves sterol-carrier protein$_2$ (SCP$_2$), although other factors are likely to be involved, including fatty acid-binding protein and other as yet unidentified factors (Strauss & Miller, 1991; Holt *et al.* 1991).

5.1.2 Steroidogenic P450 Enzymes

Three members of the cytochrome P450 enzyme superfamily are involved in ovarian steroidogenesis. All mammalian members of this group of enzymes are membrane-bound proteins with a haem prosthetic group, and they require transport of electrons from NADPH via intermediates, including one flavoprotein, for their action (Strauss & Miller, 1991; Takemori & Kominami, 1984).

Cholesterol side-chain cleavage cytochrome P450 (P450scc) is a 50kDa mitochondrial enzyme which catalyzes the initial, rate-limiting step in the synthesis of all steroids, which is the removal of six carbon atoms from the side-chain of cholesterol to produce pregnenolone, a steroid with 21 carbon atoms (C-21 steroid). Electrons from NADPH are passed via the flavoprotein ferredoxin reductase (also known as adrenodoxin reductase) to an iron-sulphur protein ferredoxin (also known as adrenodoxin) associated with ferredoxin reductase. The reduced ferredoxin dissociates from ferredoxin reductase and binds to P450scc, passing its electrons to the haem group of P450scc. Ferredoxin then is shuttled back to ferredoxin reductase. The reaction takes place in three steps; two hydroxylations at carbons 20 and 22, followed by cleavage of the bond between those atoms. Hence, the reaction requires three pairs of electrons, and therefore three cycles of reduction and oxidation of ferredoxin.

All following stages of ovarian steroidogenesis take place in the smooth endoplasmic reticulum. The synthesis of androgens (C-19 steroids) from progestogens takes place in two steps, which are both catalyzed by a 50kDa cytochrome P450 enzyme, 17α-hydroxylase/C17,20-lyase (P450c17). First, the carbon atom at position 17 is hydroxylated and second, the bond between that carbon and the carbon atom at position 20 is broken. As for the side-chain cleavage step, electrons must be provided by NADPH via a flavoprotein. Electrons for the first step are passed to P450c17 by P450 reductase, and for the second step either by P450 reductase or cytochrome b_5 (Takemori & Kominami, 1984). P450c17 can utilize either pregnenolone (a Δ^5-progestogen) or progesterone (a

Δ^4-progestogen) as a substrate, to yield dehydroepiandrosterone (DHEA) or androstenedione, respectively. However, pregnenolone is the preferred substrate (Zuber *et al.* 1986).

The third major cytochrome P450 in the ovarian steroidogenic pathway is the 55kDa aromatase enzyme (P450arom), which catalyzes the conversion of C-19 androgens to C-18 oestrogens. The reaction takes place in three steps. The methyl group at position 19 is hydroxylated twice, which causes it to be lost, and the carbon atom at position 3 is hydroxylated, leading to aromatization of the A ring. Three pairs of electrons, again derived from NADPH, are supplied by P450 reductase (Osawa *et al.* 1987). There are two major substrates for this enzyme in the ovary; androstenedione and its 17β-hydroxylated counterpart, testosterone. Action of aromatase on these steroids yields estrone and 17β-oestradiol, respectively. Aromatase is not able to use 5α-dihydrotestosterone (DHT), the most potent androgen in many tissues (Johnson & Everitt, 1988), as a substrate, and DHT can act as a competetive aromatase inhibitor (Hillier *et al.* 1980**a**; Hillier *et al.* 1980**b**).

5.1.3 Steroid Dehydrogenases

The conversion of Δ^5-steroids to Δ^4-steroids is catalyzed by the 46kDa enzyme 3β-hydroxysteroid dehydrogenase/Δ^{5-4} isomerase (3β-HSD), which also carries out 3β-hydroxylation. The enzyme is localized to the endoplasmic reticulum (Miller, 1988) in close proximity to the mitochondria, where pregnenolone is produced by the side-chain cleavage complex. This pregnenolone is converted by 3β-HSD to progesterone, using electrons donated directly by NADH or NADPH. Other substrates in the ovary include 17-hydroxypregnenolone, dehydroepiandrosterone and androstenediol, which are converted to 17-hydroxyprogesterone, androstenedione and testosterone, respectively. This step is not rate-limiting, since ovarian cells are capable of converting large amounts of pregnenolone to progesterone in the absence of trophic stimulation (Strauss & Miller, 1991).

The only readily reversible reaction in ovarian steroidogenesis is the interconversion of the 17-ketone group of dehydroepiandrosterone,

androstenedione and estrone and the 17-hydroxyl group of androstenediol, testosterone and oestradiol, respectively. Because of this, the enzyme catalyzing this step is variously called 17β-hydroxysteroid dehydrogenase (17β-HSD), 17-ketosteroid reductase or 17-oxidoreductase. This enzyme is present in both theca and granulosa cells, and appears to be constitutively expressed (Bogovich & Richards, 1984).

5.1.4 Other Ovarian Enzymes of Steroid Metabolism

Granulosa cells posess 20α-hydroxylase activity (Davoren & Hsueh, 1986), which converts progesterone to the relatively inactive progestogen, 20α-hydroxyprogesterone. They also posess 5α-reductase (Moon & Duleba, 1982; Makris & Ryan, 1980; Nimrod, 1977a), which converts testosterone to DHT. The significance of these enzymes in ovarian steroidogenesis is uncertain, but since 20α-progesterone is converted to androgen in the ovary (Goldring & Orly, 1985), it could be that the reason for the production of this progestogen is to provide substrate for androgen synthesis without the hormonal activity of progesterone.

5.2 The "Two Cell, Two Gonadotrophin" Theory

The present model of ovarian oestrogen synthesis was initially based on the observations that both FSH and LH (Fevold, 1941) and both granulosa cells and theca interna cells (Falck, 1959) are required for the synthesis of oestrogen by the ovary. In 1962, based on studies in the horse, Short proposed a "two-cell" theory whereby granulosa cells synthesize progesterone, which is then aromatized to oestrogens in the theca. For a long time, the theca were considered to be the major site of oestrogen synthesis (Ryan & Petro, 1966; Goldenberg *et al.* 1973; Makris & Ryan, 1975), although granulosa cells were shown to possess aromatase activity, as well as 17β-HSD and 3β-HSD (Bjersing, 1967). In later studies it began to emerge that granulosa cells produce oestrogen from exogenous androgen precursor in response to FSH (Dorrington *et al.* 1975; Tsang *et al.* 1987), and that in many species the granulosa cells are in fact the major, if not sole, ovarian source of oestrogen (Hillier *et al.* 1981). Conversely, it was

demonstrated that theca cells secrete androgens under the influence of LH in culture, whereas granulosa cells are unable to synthesize androgens (Tsang *et al.* 1979b; Tsang *et al.* 1987), consistent with earlier evidence that granulosa cells display very little 17α-hydroxylase activity (Bjersing, 1967). The "two-cell, two-gonadotrophin" model of ovarian oestrogen synthesis which has now gained almost universal acceptance states that LH and FSH stimulate progesterone synthesis in the theca and granulosa cells, respectively, which then acts as a substrate for LH-stimulated androgen synthesis in the theca cells. Thecal androgen diffuses across the basement membrane to the granulosa cell layer, where FSH stimulates its conversion to oestrogen. This theory is summarised in Fig.1.2.

Numerous studies in several species have now confirmed by immunocytochemistry (Yoshinaga Hirabayashi *et al.* 1987; Sasano *et al.* 1989a; Sasano *et al.* 1989b; Rodgers *et al.* 1986a) and by using cDNA probes to P450c17 mRNA (Voutilainen *et al.* 1986) that thecal and interstitial cells are the only ovarian cells which express this enzyme. The synthesis of androgens by thecal cells in culture is stimulated by LH and cAMP analogues (Tsang *et al.* 1979b; Roberts & Skinner, 1990b), and this is associated with an increase in expression of the mRNA and the protein for P450c17 (Magoffin, 1989; McAllister *et al.* 1989). Furthermore, immunocytochemical studies (Ishimura *et al.* 1989; Sasano *et al.* 1989b; Naganuma *et al.* 1990) and the use of cDNA probes for P450arom (McAllister *et al.* 1989; Hickey *et al.* 1990) have confirmed that the expression of aromatase is restricted to the granulosa cell layer, and that its expression is stimulated by FSH or artificial elevation of intracellular cAMP levels (Steinkampf *et al.* 1987).

Both cell types express P450scc (Zlotkin *et al.* 1986; McAllister *et al.* 1989) and are able to synthesize pregnenolone and progesterone in culture (Roberts & Skinner, 1990b; Hsueh *et al.* 1984). Secretion of progestogens and expression of P450scc protein and its mRNA correlate well, and are stimulated by FSH in granulosa cells, by LH in theca cells and by cAMP analogues in both cell types (Trzeciak *et al.* 1986; McMasters *et al.* 1987; Goldring *et al.* 1987; Magoffin, 1989; McAllister *et al.* 1989; Oonk *et al.* 1990; Urban *et al.* 1991). However, since a partial barrier between the blood and the granulosa cell layer exists which may limit

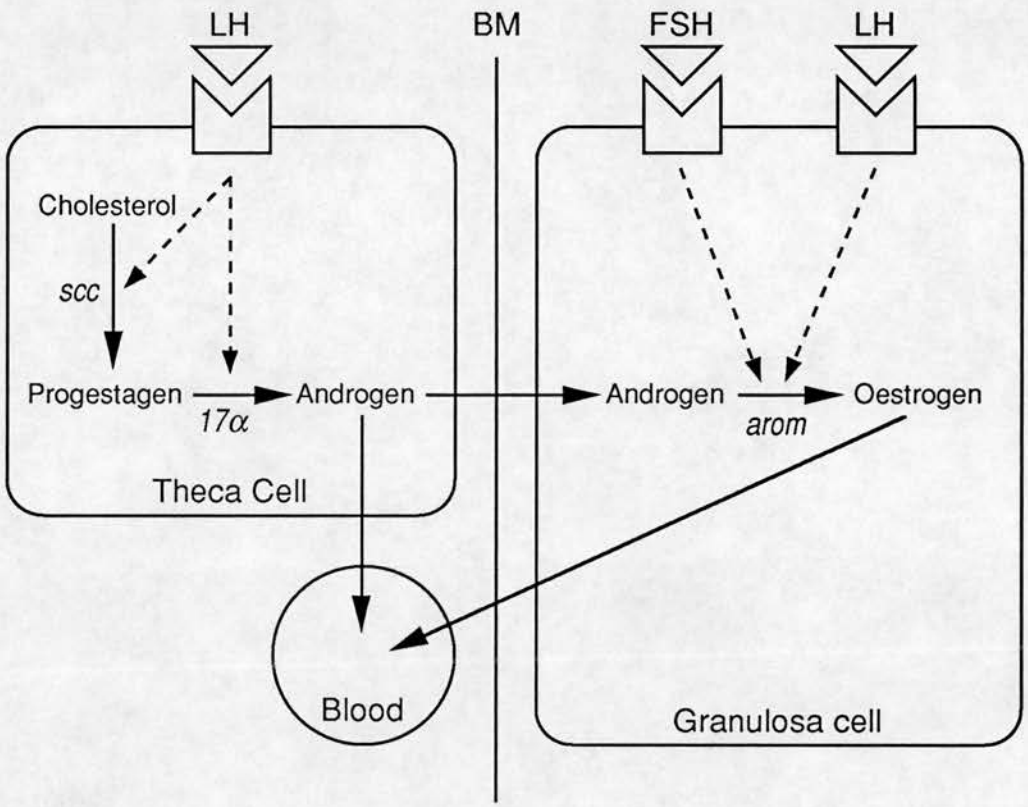


Fig.1.2. The "Two-Cell, Two-Gonadotrophin" theory of ovarian steroidogenesis. LH, acting on the theca cells, stimulates expression of the cytochrome P450 enzymes side-chain cleavage (*scc*) and 17α-hydroxylase (*17α*), leading to androgen production. Androgen diffuses across the basement membrane (BM) to the granulosa cell layer, where it is converted to oestrogen under the influence of FSH, by cytochrome P450 aromatase (*arom*). As the granulosa cells mature, they develop receptors for LH, and so oestrogen synthesis also comes under the control of LH.

access of granulosa cells to lipoproteins (Shalgi *et al.* 1973), it is uncertain whether granulosa cells within the follicle synthesize much C-21 steroid to act as precursor for androgen and oestrogen synthesis. As noted above, however, granulosa cells may synthesize cholesterol from acetate by the action of HMG CoA reductase.

Other components of the steroidogenic machinery are affected by FSH and LH which, together with increased expression of P450 enzymes, increase the steroidogenic capacity of the cells. Thecal 3β-HSD activity and mRNA levels have been shown to increase in response to hCG stimulation in the rat and pig (Martel *et al.* 1990b; Chedrese *et al.* 1990; Martel *et al.* 1990a), and granulosa cell 3β-HSD activity is stimulated by FSH (Hsueh *et al.* 1984). Levels of this enzyme mirror those of P450scc, with expression being higher in thecal cells than granulosa cells (Dupont *et al.* 1990), and highest in the corpus luteum (Zhao *et al.* 1991; Couet *et al.* 1990).

Stimulation of steroidogenic P450 enzymes by gonadotrophins is associated with some increase in the expression of their associated electron transport proteins, reflecting an increase in the number of mitochondria (Rodgers *et al.* 1986b). Expression of ferredoxin in human preovulatory granulosa cells is increased by hCG, FSH and cAMP analogues (Voutilainen *et al.* 1988). It has also been shown in the rat that follicular ferredoxin mRNA levels rise in response to an ovulatory dose of hCG, albeit transiently (Hedin *et al.* 1987b). In the same study, no change in P450 reductase expression was observed. Studies in the cow have shown that ferredoxin mRNA levels are at their highest in the corpus luteum (Rodgers *et al.* 1987b). The relationship between expression of steroidogenic P450 enzymes and their electron donors is not yet clear, presumably because these proteins are generic electron transport components involved in all P450-mediated processes.

Also, the dynamics of cholesterol synthesis and uptake, and accumulation and breakdown of intracellular cholesterol ester stores are affected by gonadotrophins. LDL receptor expression is stimulated in mature human granulosa cells by hCG and cAMP (Golos *et al.* 1986; Golos *et al.* 1987; Golos & Strauss, 1987). In the bovine ovary LDL receptor expression is highest in the corpus luteum (Rodgers *et al.* 1987b), when

the highest level of steroidogenesis is seen. HMG CoA reductase expression is also stimulated by cAMP in the bovine corpus luteum (Rodgers *et al.* 1987a), and transiently in the rat follicle following hCG stimulation (Hedin *et al.* 1987b). The dynamics of cholesterol ester synthesis and hydrolysis are affected by gonadotrophin stimulation. However, these effects may not be direct, but due to removal of intracellular feedback effects of oxysterol (Strauss & Miller, 1991).

These observations generally correlate well with the fluctuations in the expression of the three key enzymes in the ovary during the cycle. P450c17 and P450scc expression can be detected in the theca layer of atretic as well as healthy follicles from the early antral stage onwards (Zlotkin *et al.* 1986; Rodgers *et al.* 1986a; Hedin *et al.* 1987b). Aromatase is not expressed in granulosa cells of small follicles, but is expressed in large antral follicles (Hickey *et al.* 1988; Ishimura *et al.* 1989; Sasano *et al.* 1989b). There is some disagreement concerning the exact stage at which P450scc is first expressed in granulosa cells, but it is clear that it is not expressed in these cells until at least the late preovulatory stage (Rodgers *et al.* 1986a; Zlotkin *et al.* 1986; Le Goascogne *et al.* 1989). At the LH surge, P450scc increases still further (Doody *et al.* 1990; Hedin *et al.* 1987b), and there is evidence that this enzyme is expressed constitutively in the corpus luteum (Oonk *et al.* 1990; Hedin *et al.* 1987b). Androgen production is rapidly suppressed at the LH surge, probably due to increased progestogen production which inhibits P450c17 activity (Eckstein *et al.* 1985; Johnson, 1988), and in most species, with the exception of the human and other primates (Doody *et al.* 1990) this is followed by a decline in P450c17 mRNA and protein (Ishimura *et al.* 1990; Hedin *et al.* 1987b; Rodgers *et al.* 1986b). P450arom is present in low abundance in the corpus luteum of many species, including ones which do not secrete oestrogen during the luteal phase (Yoshinaga Hirabayashi *et al.* 1990; Hickey *et al.* 1988). It is therefore likely that in these species the corpus luteum does not secrete oestrogen due to lack of androgen substrate, rather than absence of aromatase. In human ovaries, P450arom mRNA is more abundant in the corpus luteum than in preovulatory follicles (Doody *et al.* 1990). This is surprising, since plasma oestradiol levels are at their highest during the late follicular phase. This may be due to the large amounts of

progesterone produced in the luteal phase, which may inhibit P450c17, limiting production of androgen substrate (Eckstein *et al.* 1985; Johnson, 1988).

6 Local Control of Follicle Development

All follicles are exposed to the same blood levels of gonadotrophins, and yet most which begin to grow become atretic, while others become dominant and ovulate. This strongly suggests that factors other than gonadotrophins act locally to bring about this difference in the fate of follicles. The critical factors determining the fate of a follicle are believed to be the responsiveness of its granulosa cells to FSH and their ability to respond to LH i.e. their degree of differentiation. Therefore, a great deal of research has been carried out into the effects of various locally produced steroids, proteins and other factors on granulosa cell differentiation.

6.1 Role of Steroids

Since the most conspicuous secretory products of follicular cells are steroids, their role for these factors in the local control of granulosa cell differentiation has been studied in some detail.

6.1.1 Mechanism of Action of Steroids

Steroid action, according to current concepts, is mediated by specific receptors which belong to a family of transcription factors. Following cloning and sequence comparison, it has been found that steroid receptors belong to a large family of nuclear hormone receptors which includes the receptors for thyroid hormones, vitamin D_3, and retinoic acid, as well as some proteins for which no ligand has yet been found (Evans, 1988; Parker, 1988). These nuclear hormone receptors have a highly modular structure, with distinct domains fulfilling discrete functions (see Fig.1.3) which have been elegantly characterised by mutagenesis (Kumar *et al.* 1986; Jenster *et al.* 1991) and generation of chimaeric receptor proteins (Green & Chambon, 1987; Green & Chambon, 1989). The most highly

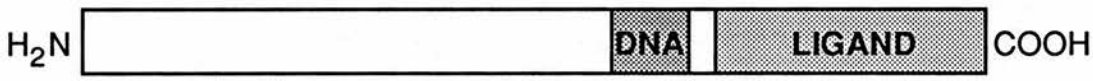


Fig. 1.3. General structure of members of the nuclear hormone receptor superfamily, including steroid receptors. "DNA" denotes the DNA-binding domain, and "LIGAND" denotes the ligand-binding domain.

conserved domain of these receptors is the DNA-binding domain, which is rich in cysteine residues (Green & Chambon, 1986). These residues act to coordinate zinc ions, forming secondary structures known as zinc fingers (Evans & Hollenberg, 1988; Berg, 1990), similar to those found in some other transcription factors (Struhl, 1989; Harrison, 1991; Miller *et al.* 1985). Steroid receptors possess two zinc fingers which are capable of binding to specific short palindromic DNA sequences in the genome known as hormone response elements (HREs) (Ham *et al.* 1988; Beato *et al.* 1989; Umesono & Evans, 1989), which act as enhancers (Ptashne, 1988). Binding of a steroid receptor to its HRE leads to enhancement of transcription of the downstream gene, by interacting with other transcription factors at the gene promoter (Schule *et al.* 1988a; Schule *et al.* 1988b; Beato, 1989; Klein Hitpass *et al.* 1990), and/or by causing a change in chromatin structure such that these other factors may bind (Spelsberg *et al.* 1989; Beato, 1989). The carboxy-terminal steroid-binding domain is less highly conserved between members of this family, and the degree of homology between receptors roughly reflects the difference in structure of their ligands (Evans, 1988). This region is thought to act to repress the transcriptional activation activity of the receptor in the absence of hormone (Webster *et al.* 1988; Hollenberg *et al.* 1987; Godowski *et al.* 1987), and to be involved in dimerisation of occupied receptors (Fawell *et al.* 1990). The function of the N-terminal region of these receptors, which is highly variable in size and sequence, is not clear, but it is required for the full activity of the receptor (Bocquel *et al.* 1989; Lees *et al.* 1989).

Except for the glucocorticoid receptor (Gustafsson *et al.* 1989; Gustafsson *et al.* 1987), steroid receptors are located predominantly in the nuclei of target cells (Martin & Sheridan, 1986; Gasc & Baulieu, 1986; King, 1987), and are bound to a number of proteins in their inactive state, in particular a 90kDa heat-shock protein (hsp90) (Ziemiecki *et al.* 1986; Joab *et al.* 1984). This protein is thought to prevent activation of the receptor in the absence of steroid (Cadepond *et al.* 1991), by binding to several sites of the receptor (Carson Jurica *et al.* 1989; Chambraud *et al.* 1990), including the DNA-binding domain (Baulieu, 1987; Smith *et al.* 1990a), and preventing dimerisation of receptors (DeMarzo *et al.* 1991).

Upon ligand binding, the receptor becomes activated, or "transformed", by a process involving dissociation of hsp90 (Denis & Gustafsson, 1989; Nakao & Moudgil, 1989; DeMarzo *et al.* 1991), and receptor dimerisation (Tsai *et al.* 1989; O'Malley, 1990). Activation of steroid receptors is also thought to involve phosphorylation of the receptor (Auricchio, 1989; Orti *et al.* 1992), although the significance of this is still not fully clear. In this active state, hormone-receptor complexes bind as dimers, via their zinc-finger domains, to their HREs and enhance transcription of hormone-responsive genes (Beato, 1989).

6.1.2 Oestrogens

The involvement of oestrogen in the local as well as endocrine control of follicle development has long been suspected. Injection of oestrogen to rats reduces the number of follicles undergoing atresia (Ingram, 1959a; Ingram, 1959b; Harman *et al.* 1975b; Richards, 1975), increases proliferation of granulosa cells (Goldenberg *et al.* 1972), and augments the stimulation of ovarian weight and follicle development caused by FSH (Simpson *et al.* 1941; Goldenberg *et al.* 1972; Richards, 1975). The increased responsiveness to FSH is accompanied by an increase in the number of receptors for FSH in the granulosa cells of oestrogen-treated animals. Furthermore, administration of specific antibodies to oestradiol or the antioestrogen clomiphene inhibits the effects of FSH and LH on follicle development and ovarian weight (Harman *et al.* 1975a; Reiter *et al.* 1972). Indeed the most widely used granulosa cell culture model makes use of the effect of oestrogen when administered *in vivo* on the proliferation of rat granulosa cells to increase yields of granulosa cells (Dorrington *et al.* 1975; Sanders & Midgley, 1982; Hsueh *et al.* 1984). Oestrogen administered *in vivo* also increases communication between granulosa cells via increased homologous gap junction formation (Merk *et al.* 1972).

In addition to its effects on granulosa cell proliferation, which is probably an indirect effect (see above), a number of the effects of FSH on the differentiation of granulosa cells have been shown to be augmented by simultaneous administration of oestrogens. Oestrogen augments the

induction of aromatase activity stimulated by FSH (Merk *et al.* 1972; Daniel & Armstrong, 1983; Adashi & Hsueh, 1982), thereby stimulating its own synthesis. It also augments the stimulation by FSH of progesterone production by cultured granulosa cells (Welsh *et al.* 1983; Fortune & Hansel, 1979; Ratoosh & Richards, 1985), although it is inhibitory in several species at high concentrations (Thanki & Channing, 1976; Shemesh & Ailenberg, 1977; Bieszczad *et al.* 1982), probably due to direct inhibition of the 3β-HSD enzyme (Veldhuis *et al.* 1983). The stimulation of progesterone production is due to increased levels of P450scc protein (Toaff *et al.* 1983), although oestradiol appears to have no effect on basal or FSH-stimulated levels of P450scc mRNA in pig granulosa cells (Urban *et al.* 1990; Urban *et al.* 1991). The other major marker for FSH-stimulated differentiation of granulosa cells is the acquisition of LH receptors, and this too has been shown to be augmented by oestrogens (Richards *et al.* 1976; Sheela Rani *et al.* 1981; Knecht *et al.* 1985). These effects of oestrogen appear to be specific, since they can be blocked with specific antioestrogens (Veldhuis *et al.* 1986b; Olsson & Granberg, 1990), and the effects of FSH or cAMP analogues can be inhibited with antioestrogens (Knecht *et al.* 1985; Kessel & Hsueh, 1987), presumably by blocking the action of endogenous oestrogen produced in response to FSH. Interestingly, oestrogens appear to have no effect on the ability of FSH to increase numbers of its own receptor, although oestrogen treatment alone does increase numbers of FSH receptors (Richards *et al.* 1976). It has also been suggested that FSH stimulation of proteoglycan production is under the modulatory influence of oestrogen (Schweitzer *et al.* 1981), although in another study no effect was seen (Yanagishita *et al.* 1981).

The augmentation by oestrogen of a number of FSH-stimulated parameters of granulosa cell function is probably mediated, at least in part, at the level of cAMP generation or breakdown. Both *in vitro* and *in vivo*, oestrogens have been shown to amplify the levels of cAMP generated in granulosa cells in response to FSH (Richards *et al.* 1979; Jonassen *et al.* 1982). This is presumably the basis for the augmentation of the effects of FSH by oestradiol. The mechanism by which oestrogens enhance FSH-stimulated cAMP generation has not been extensively studied, but it does not seem to involve an increase in FSH receptor

numbers, nor inhibition of cAMP breakdown by phosphodiesterases (Richards *et al.* 1979). Also, oestrogen is capable of augmenting the effects of forskolin, cholera toxin and 8-bromo-cAMP on LH receptor, FSH receptor and cAMP accumulation in cultured granulosa cells (Knecht *et al.* 1985; Knecht *et al.* 1984). The effect of oestrogen on cAMP accumulation is seen only after some time in culture (Knecht *et al.* 1984), suggesting that oestrogen does inhibit breakdown of cAMP. The effects of oestrogen on gonadotrophin-stimulated cAMP production are fairly modest in comparison to the very marked augmentation of progesterone, oestrogen and LH receptor production (Hudson *et al.* 1987; Adashi & Hsueh, 1982; Knecht *et al.* 1984; Knecht *et al.* 1985), suggesting that another mechanism may be involved, although the response to cAMP levels may not of course be linear.

Because of this rather generalized effect of oestrogen, many effects of FSH are augmented by oestrogen. Such effects include expression of a regulatory subunit (RIIβ) of protein kinase A. The precise significance of this finding is not known, since the pattern of expression of the other subunits of protein kinase A does not follow the same pattern (Richards *et al.* 1987), but it may indicate an increased intracellular capacity to respond to cAMP. Increased sensitivity to FSH in generating intracellular cAMP, coupled to an increased ability to respond to that messenger, may constitute a highly efficient mechanism whereby the threshold level of FSH required for the continued development of the follicle could be reduced by oestrogen. However, increased levels of RII do not increase sensitivity of cells to cAMP (Richards & Hedin, 1988), and one would expect that increased levels of this protein, which suppresses the activity of the catalytic subunits of protein kinase A, would lead to reduced rather than increased cAMP responsiveness.

Oestrogen may also have paracrine effects on thecal androgen production. Studies in pigs (Tsang *et al.* 1979a; Tonetta & Hernandez, 1989) and rats (Leung & Armstrong, 1979; Magoffin & Erickson, 1982) have shown that oestrogens strongly inhibit androgen production, although a study using cow theca cells showed a stimulatory effect of oestrogen (Roberts & Skinner, 1990a). The inhibitory action of oestrogen on androgen production is not exerted through altered generation of

cAMP by theca cells (Morley *et al.* 1989; Hunter & Armstrong, 1987), and is probably due to direct inhibition of the P450c17 enzyme (Leung & Armstrong, 1979; Magoffin & Erickson, 1982; Gower & Cooke, 1983).

In order that oestrogens may exert their effects, conventional ideas would dictate that they do so via interaction with specific oestrogen receptors. Specific, high affinity binding sites for oestrogens have been identified in the ovaries of rats and hamsters (Kim & Greenwald, 1987; Saiduddin & Zassenhaus, 1977), most of which appear to be in the granulosa cells (Richards, 1975; Kudolo *et al.* 1984b; Kudolo *et al.* 1984a). These high affinity binding sites are located in the nucleus of these cells (Kudolo *et al.* 1984b; Richards, 1975), and specific nuclear uptake of intracellularly produced oestradiol has been demonstrated in cultured rat granulosa cells (Wolfson *et al.* 1990). Furthermore, the abundance of nuclear binding sites for oestrogen is increased by treatment of animals with oestrogen, unchanged by treatment with FSH or progesterone, and reduced by treatment with LH or androgen (Richards, 1975; Saiduddin & Zassenhaus, 1978). However, immunocytochemical studies are scarce and contradictory on the existence of typical oestrogen receptors in granulosa cells. In monkey ovaries, oestrogen receptor has been detected *only* in the germinal epithelium (Hild Petito *et al.* 1988), whereas in human ovaries, positive staining for the oestrogen receptor has been found in the granulosa cells of large antral follicles prior to the LH surge (Iwai *et al.* 1990). Studies using nucleic acid probes have failed to detect oestrogen receptor mRNA in mouse granulosa cells (Hillier *et al.* 1989a). Intriguingly, several studies have indicated that there are oestrogen receptor-related molecules present in granulosa cells, as there are in several other tissues (Ataya *et al.* 1988; Giguere *et al.* 1988). Kudolo *et al.* (1984b) reported a second oestrogen-binding species in the cytoplasm of rat granulosa cells. This protein has a much lower affinity for oestradiol than does the nuclear species (46nM, compared to 0.6nM K_d), and also binds androgens and progestogens (although with lower affinity than for oestradiol). Also, the amount of cytoplasmic oestrogen binding is elevated after treatment of animals with FSH and LH (Kudolo *et al.* 1987). Oestrogen receptor-related mRNA has also been detected in high abundance in mouse ovary using RNA probes complementary to the

region of the oestrogen receptor mRNA which encodes the steroid binding domain of the receptor (Hillier *et al.* 1989a). This smaller (~1.5 kilobases, compared to ~6.5 kilobases) message is confined to the granulosa cells, is not detected with RNA probes corresponding to other regions of the authentic oestrogen receptor mRNA, and appears not to be affected by gonadotrophin treatment. Furthermore, this species is not generated from the oestrogen receptor gene by alternative splicing, since it hybridizes less strongly to the oestrogen receptor RNA probe than does the authentic uterine oestrogen receptor mRNA. The function of these species is uncertain, but they could be intracellular oestrogen-binding proteins which control the levels of free oestrogen within the cell. Meanwhile the existence of true oestrogen receptors in granulosa cells remains in doubt.

6.1.3 Androgens

Androgens produced by the theca and interstitial cells do not simply act as a substrate from which granulosa cells synthesize oestrogens, but also have a hormonal role in the local control of follicular development. Initial evidence suggested that androgens promote atresia, and so an action of androgen antagonistic to that of oestrogen was proposed. Administration of androgens to hypophysectomized animals *in vivo* does lead to a reduction in ovarian weight and wholesale atresia of follicles (Hillier & Ross, 1979). In addition, it is now clear that androgens are capable of modulating the action of FSH on granulosa cells in much the same way as oestrogens. However, the function of androgens *in vivo* is not certain, since administration of antiandrogens does not disrupt rat oestrous cycles (Neumann *et al.* 1970) nor does it affect FSH responsiveness in rats (Zeleznik *et al.* 1979), and androgen resistant mice have normal cycles (Lyon & Glenister, 1974). On the other hand, intraovarian implants of antiandrogen reduce progesterone secretion by granulosa cells in subsequent culture (Schomberg *et al.* 1978).

Many studies have now shown that FSH-stimulated progesterone and oestradiol synthesis by granulosa cells *in vitro* is greatly enhanced by the addition of androgens to the medium. This is true for granulosa cells

isolated from small follicles in the rat (Hudson *et al.* 1987; Nimrod, 1977a; Nimrod, 1981; Lucky *et al.* 1977; Armstrong & Dorrington, 1976; Duleba *et al.* 1984), pig (Spicer & Hammond, 1988b), cow (Luck *et al.* 1990; Henderson & Franchimont, 1983), sheep (Moor *et al.* 1975) and monkey (Harlow *et al.* 1986; Shaw *et al.* 1989). Androgens also augment gonadotrophin-stimulated progesterone production in mature granulosa cells (Zeleznik *et al.* 1979; Quirk & Fortune, 1983; Corredor & Flickinger, 1983; Haney & Schomberg, 1978). Oestrogen production stimulated by FSH or cAMP analogues is also augmented by androgens (Daniel & Armstrong, 1980; Hillier & deZwart, 1982; Shaw *et al.* 1989). The possibility that these effects may be mediated by oestrogen following aromatization of the androgen has been ruled out by several pieces of evidence. Firstly, androgens such as testosterone and androstenedione are far more potent than oestrogen in the augmentation of FSH-stimulated steroid production (Hillier *et al.* 1985; Armstrong & Dorrington, 1976; Duleba *et al.* 1985). Secondly, nonaromatizable androgens such as 5α-dihydrotestosterone (DHT) are very nearly as potent as testosterone in this respect (Hillier & deZwart, 1981; Hillier *et al.* 1988; Spicer & Hammond, 1988b). Thirdly, the effect of androgens can be blocked with a specific antiandrogen, whereas that of oestrogen cannot (Duleba *et al.* 1985; Spicer & Hammond, 1988b; Hudson *et al.* 1987; Hillier & deZwart, 1982). Conversely, the effects of oestrogen can be blocked with antioestrogen (Veldhuis *et al.* 1986b; Knecht *et al.* 1985), while those of androgen cannot (Daniel & Armstrong, 1983). For more mature granulosa cells (i.e. from larger follicles), androgen may in fact be inhibitory (Polan *et al.* 1986; Hillier *et al.* 1988). Androgens also enhance many other of the effects of FSH associated with granulosa cell differentiation, including the development of LH responsiveness (Shaw *et al.* 1989), inhibin production (Hillier *et al.* 1989b; Michel *et al.* 1991b; Henderson & Franchimont, 1981; Henderson & Franchimont, 1983), proteoglycan production (Yanagishita *et al.* 1981) and plasminogen activator secretion (Wang & Leung, 1987**a; Jia et al. 1990**).

The mechanism by which androgens exert their modulatory effects on FSH-stimulated granulosa cell differentiation is not fully understood. Androgens, like oestrogens, are capable of enhancing levels of cAMP

generated in response to FSH (Hillier & deZwart, 1982; Knecht *et al.* 1984; Goff *et al.* 1979). However, Nimrod (1977b) observed no change in cAMP generation or breakdown associated with androgen augmentation of FSH-stimulated progestogen production relative to FSH alone. Furthermore, like oestrogens, androgens can augment the stimulation of progesterone and oestrogen synthesis brought about by the cAMP analogue, 8-bromo cAMP and also by cholera toxin (Hillier & deZwart, 1982; Nimrod, 1977b; Knecht *et al.* 1984). As is the case for oestrogen, androgens enhance FSH-stimulated steroidogenesis to a much greater degree than cAMP accumulation (Hillier & deZwart, 1982; Welsh *et al.* 1982), so there may be other mechanisms involved, such as modification of the temporal pattern of, or sensitivity to, cAMP generation.

The demonstration that androgen effects on granulosa cells can be blocked with specific antiandrogens (Duleba *et al.* 1985; Hudson & Hillier, 1985; Spicer & Hammond, 1988b; Hudson *et al.* 1987; Hillier & deZwart, 1982) suggests that these effects are receptor-mediated. High affinity (K_d 2-3nM) binding sites specific for testosterone and distinct from androgen-binding protein have been found in rat (Schreiber *et al.* 1976) and human (Milwidsky *et al.* 1980) ovaries. Furthermore, nuclear uptake of labelled testosterone has been demonstrated in rat ovaries (Zeleznik *et al.* 1979; Schreiber & Ross, 1976), which can be inhibited by antiandrogen (Zeleznik *et al.* 1979). These binding sites have a greater affinity for testosterone than other androgens. Indeed, the affinity of the rat ovarian androgen "receptor" is no greater for DHT than for oestradiol (Schreiber *et al.* 1976). These characteristics may explain why testosterone is more potent than DHT in augmenting the actions of FSH (Hillier & deZwart, 1982), but does not explain why DHT is more potent than oestradiol in this respect (Hudson *et al.* 1987). The cellular distribution of ovarian androgen receptor is still not clear since, as with oestrogen receptor studies, recent immunocytochemical studies have been inconclusive. In the rat ovary, Takeda *et al.* (1990) found weak positive staining for androgen receptor only in granulosa-lutein cells, whereas in the monkey ovary, staining has been observed in virtually every cell type in the ovary (Hild Petito *et al.* 1991). It appears likely that the effects of androgens on granulosa cells are receptor-mediated, but until the existence of conventional androgen

receptors in these cells is unequivocally demonstrated, the mechanism of androgen action remains unknown.

6.1.4 Progestogens

As well as androgens and oestrogens, progestogens, in particular progesterone, have also been implicated in the local control of follicular and luteal function, although the evidence to support such a role is less clear-cut than for androgens and oestrogens. Regression or removal of the corpus luteum is followed by commencement of antral follicular growth (Goodman & Hodgen, 1979; Baird *et al.* 1984; Mills & Stopper, 1989), although large preantral and even small antral follicles continue to develop in the presence of an active corpus luteum (McNatty *et al.* 1983; Baird *et al.* 1984; Zeleznik *et al.* 1980). Also, antral follicular growth is inhibited by administration of exogenous progesterone to intact animals (Buffler & Roser, 1974; Greenwald, 1977). These observations have led to a hypothesis that progesterone exerts an inhibitory influence upon follicle development (Mills & Stopper, 1989; Goodman & Hodgen, 1983), at least during the luteal phase. However, these results have been explained in terms of negative feedback of gonadotrophin secretion by progesterone, rather than direct effects of progesterone on the ovary (Taya *et al.* 1981; Garza & Terranova, 1984). This explanation is supported by the finding that exogenous gonadotrophin is able to stimulate follicle growth in ovaries containing an active corpus luteum (Zeleznik & Resko, 1980). In fact, it has been shown that the effect of hCG on the growth of small follicles is augmented by elevated serum progesterone levels brought about by the use of progesterone implants in intact animals (Bogovich *et al.* 1981; Richards & Bogovich, 1982; Bogovich & Richards, 1982), although the serum levels of progesterone attained by this method were very high. In hypophysectomized rats, treatment with progesterone alone caused no change in the morphology of the ovary in a number of studies (Richards, 1975; Saiduddin & Zassenhaus, 1978; Smith & Bradbury, 1966), although it has been shown to inhibit follicular growth brought about by oestrogen (Smith & Bradbury, 1966; Payne *et al.* 1956) and gonadotrophin (Chiras & Greenwald, 1978).

In vitro studies on the direct effects of progesterone on ovarian cells have yielded scarcely more conclusive results. Stimulatory effects of progesterone (or a synthetic analogue, R5020) on cultured granulosa cells have been observed, with respect to gonadotrophin-stimulated progesterone production (Ruiz de Galarreta *et al.* 1985; Fanjul *et al.* 1983) and plasminogen activator (Wang & Leung, 1987a), both markers of advanced granulosa cell development. These effects can be blocked by the antiprogestin RU486 (Wang & Leung, 1987a), and cannot be blocked with an antiandrogen (Fanjul *et al.* 1983). The effect of R5020 on FSH-stimulated progesterone production was very modest and exerted only at very high doses, but its augmentation of LH action was much greater (Fanjul *et al.* 1983), supporting the notion that progesterone begins to be important only in the later stages of granulosa cell development. Studies on cultured hamster ovaries suggest that progesterone may also promote ovulation (Baranczuk & Fainstat, 1976), possibly by interfering with synthesis of connective tissue, leading to thinning of the follicle wall (Tjugum *et al.* 1984). Inhibitory effects of progesterone on cultured granulosa cells have also been observed. Progesterone inhibits FSH-stimulated oestrogen production in cultured rat granulosa cells (Schreiber *et al.* 1980; Schreiber *et al.* 1981; Fortune & Vincent, 1983) and rabbit follicles (Bahr *et al.* 1980), although in a study by Daniel and Armstrong (1980), no effect of progesterone on oestrogen synthesis was observed. Progesterone also inhibits induction of LH receptors in rat granulosa cells (Schreiber *et al.* 1982), and may (Henderson & Franchimont, 1981) or may not (Michel *et al.* 1991b) inhibit inhibin production in mature cow and pig granulosa cells, respectively. It has been pointed out that, since rather high concentrations of progesterone are required to exert significant effects, and since high concentrations of progesterone do not develop in the follicle until preovulatory development (McNatty *et al.* 1975) by which time aromatase activity and LH receptors are already induced, these effects of progesterone can be of little importance (Hillier, 1985).

Data regarding the effects of progesterone on thecal cells is very scarce. Androgen production by pig thecal cells is affected by progesterone depending on the size of the follicles from which they were obtained, being inhibited in cells from medium-sized follicles, and stimulated in

cells from large follicles (Tonetta & Hernandez, 1989). Androgen production by thecal cells from medium-sized pig follicles is moderately enhanced by incubation in granulosa cell-conditioned medium (Lischinsky & Armstrong, 1983). It was found that substitution of progesterone and pregnenolone in the concentrations present in the conditioned medium was able to elicit a response in thecal cells equivalent to that elicited by granulosa cell-conditioned medium. The authors suggested on the basis of these results that granulosa cells pass progestogens to the thecal cells, which act as androgen substrate. This may not be the only way in which progestogens affect thecal androgen production, since P450scc immunostaining in thecal cells of small rat follicles is increased by administration of the antiprogestin RU486 (Le Goascogne *et al.* 1989).

The mechanism of direct actions of progesterone on granulosa cells is not known. It has been suggested that its effects on oestrogen production are exerted subsequent to cAMP generation (Schreiber *et al.* 1981), which may explain why progesterone can exert inhibitory effects at the same time as augmenting (albeit modestly) FSH-stimulated cAMP production (Goff *et al.* 1979). It has been suggested that some effects of high concentrations of progesterone and R5020 may be mediated through the androgen receptor (Hillier, 1985), since R5020 has been shown to exhibit androgenic actions in some tissues (Bardin & Catterall, 1981), and since progesterone can be converted to 5α-reduced androgen within the ovary (Inaba *et al.* 1978). However, the fact that the action of progesterone can be blocked with antiprogestins (Wang & Leung, 1987a), and not with antiandrogens (Fanjul *et al.* 1983) suggests that these actions are mediated through the progesterone receptor, at least in part. Moderately high affinity binding sites for progesterone have been identified in whole ovary extracts of the rat (Fujii Hanamoto *et al.* 1985; Schreiber & Hsueh, 1979; Schreiber *et al.* 1983) and human (Milwidsky *et al.* 1980), and in rat granulosa cells (Schreiber & Erickson, 1979; Naess, 1981). The levels and affinity of these receptors appears to vary with the oestrous cycle (Fujii Hanamoto *et al.* 1985), but the significance of the change in affinity is unclear. More recently, immunocytochemical studies have established that in human and monkey ovaries, progesterone receptor is present in

theca cells, interstitial cells and stroma throughout the menstrual cycle, but only appears in granulosa cells after the LH surge (Iwai *et al.* 1990; Hild Petito *et al.* 1988).

The available evidence, therefore points to there being little effect of progesterone in the early stages of follicle development, when there is little progesterone or progesterone receptor present in any case. This steroid may well be important in ovulation, and in autoregulation of progesterone production in the corpus luteum. The seemingly contradictory stimulatory and inhibitory effects of progesterone on different parameters may reflect the well known complexity with which progesterone interacts with oestrogen (Mester & Baulieu, 1984). The known effect of oestrogen on induction of progesterone receptors in other tissues may explain the observed pattern of progesterone receptor in granulosa cells of preovulatory (oestrogenic) follicles (Iwai *et al.* 1990; Hild Petito *et al.* 1988).

6.1.5 Catecholoestrogens

Another class of steroids which may have intraovarian functions are the catecholoestrogens. These steroids are formed by hydroxylation of oestrogens at the 2- or 4-positions by oestrogen-2/4-hydroxylases. In the uterus the major product is 4-hydroxyoestradiol (4-OH-E_2), and in the conceptus and ovary 2-hydroxyoestradiol (2-OH-E_2) is produced in larger quantities. It has been suggested that these steroids participate in implantation and establishment of pregnancy (Chakraborty *et al.* 1989; Paria *et al.* 1990). Catecholoestrogens have been detected in nanomolar concentrations in human and horse follicular fluid (Dehennin *et al.* 1984; Spicer & Hammond, 1989b), and in porcine follicles the enzyme oestrogen-2-hydroxylase is abundant in both theca and granulosa cells, although not in corpora lutea (Spicer & Hammond, 1989b). Furthermore, the capacity of granulosa cells to metabolize E_2 to 2-OH-E_2 increases with follicular development (Eppig & Downs, 1984). It has been shown that 2-OH-E_2 is capable of stimulating basal, gonadotrophin-stimulated and catecholamine-stimulated progesterone production by rat and pig granulosa cells (Hudson & Hillier, 1985; Hudson *et al.* 1987; Spicer &

Hammond, 1987b; Spicer & Hammond, 1987a), at the level of P450scc mRNA expression (Spicer *et al.* 1990). It has also been demonstrated that 2-OH-E_2 inhibits proliferation of pig granulosa cells (Spicer & Hammond, 1989a). Further evidence for a local function of 2-OH-E_2 came from the finding that it is also able to inhibit androgen production by pig thecal cells (Morley *et al.* 1989). It was found that pregnenolone accumulation was enhanced, while production of androstenedione and 17α-hydroxyprogesterone was reduced, suggesting that 2-OH-E_2 acts directly on the P450c17 enzyme in much the same way as does oestradiol. The mechanism by which 2-OH-E_2 exerts its effects on granulosa cells is unclear, although it is known that it acts, at least in part, at a site distal to cAMP generation, since it augments progesterone production stimulated not only by gonadotrophins, but also by cAMP analogues (Morley *et al.* 1989). The action of 2-OH-E_2 is distinct from that of oestradiol, since its potency is greater than that of oestradiol (Hudson *et al.* 1987; Spicer & Hammond, 1987a). 2-OH-E_2 does not bind to α- or β-adrenergic receptors, although it is able to increase numbers of β-adrenergic receptors in granulosa cells (Spicer & Hammond, 1988a). However, the effects of 2-OH-E_2 can be blocked with an antiandrogen, SCH16423 (hydroxyflutamide), suggesting that 2-OH-E_2 acts in a similar fashion to androgens (Hudson *et al.* 1987; Spicer & Hammond, 1988b). Altogether, these findings point to a local role for catecholoestrogens in the control of follicular development, but their exact importance is not yet known.

6.2 Role of Peptide Factors

6.2.1 IGFs

Of all the growth factors implicated in the local control of follicle development, the insulin-like growth factors (IGFs), or somatomedins, have been most extensively studied. IGFs (types I and II) are single chain polypeptides which have close homology with insulin (Froesch *et al.* 1985), and IGFs and insulin cross react to varying degrees with one another's receptors, which are present in most cells (Rechler & Nissley, 1985). IGFs produced by the liver in response to growth hormone (GH) act

as endocrine hormones acting upon various tissues (Slack, 1989). However, IGFs are not only produced by the liver, but also in other tissues where they exert their actions locally (Adashi *et al.* 1985a; Daughaday & Rotwein, 1989). The rat and pig ovary produces predominantly IGF type I (IGF-I, somatomedin-C), and of all adult tissues studied, only the uterus and liver produce more (Murphy *et al.* 1987). The fact that IGF-I concentrations much higher than those in plasma have been found in human follicular fluid (Geisthovel *et al.* 1989b; Rabinovici *et al.* 1990a) suggested an ovarian site of production. It has now been shown in the rat (Hernandez *et al.* 1989; Oliver *et al.* 1989) and pig (Hammond *et al.* 1985; Hsu & Hammond, 1987a) that the granulosa cells are the principal site of ovarian IGF-I gene expression and protein synthesis. *In vivo*, the expression of the IGF-I gene is confined to the granulosa cells of healthy antral follicles, and is greatest in the cumulus cells and the cells lining the antrum (Oliver *et al.* 1989). Although the hormonal regulation of IGF-I expression in granulosa cells has not been extensively studied, pig granulosa cell IGF-I production has been reported to be stimulated by FSH, LH, GH and oestradiol, via the cAMP second messenger pathway (Hsu & Hammond, 1987b; Hsu & Hammond, 1987a). IGF-I production by porcine granulosa cells is also modulated by a number of other growth factors (Mondschein *et al.* 1988; Mondschein & Hammond, 1988), and may actually be inhibited by FSH (Mondschein & Hammond, 1988). Receptors for IGF-I have been demonstrated in rat (Adashi *et al.* 1986a; Adashi *et al.* 1986d), sheep (Monget *et al.* 1989), pig (Veldhuis & Furlanetto, 1985) and human (Gates *et al.* 1987) granulosa cells, and in rat theca cells (Hernandez *et al.* 1988; Cara *et al.* 1990; Cara & Rosenfield, 1988). Expression of granulosa cell IGF-I receptors is also stimulated by FSH, LH and GH *in vivo* and *in vitro* (Adashi *et al.* 1988b; Adashi *et al.* 1986a), again through cAMP production.

The intraovarian function of IGF-I is not fully understood since, although it promotes proliferation of granulosa cells in some species (see above), it also augments the effects of FSH on a number of the differentiated functions of granulosa cells without any effect on proliferation (Adashi *et al.* 1985a). IGF-I augments FSH-stimulated progesterone production by cultured rat (Adashi *et al.* 1985b; Davoren *et*

al. 1985; Hutchinson *et al.* 1988), cow (Schams *et al.* 1988) and pig (Veldhuis & Furlanetto, 1985; Maruo *et al.* 1988) granulosa cells, due to increased levels of P450scc mRNA (Veldhuis *et al.* 1986a; Urban *et al.* 1990) and protein (Veldhuis & Rodgers, 1987; Veldhuis *et al.* 1986b). FSH-stimulated oestrogen synthesis is also stimulated by IGF-I (Davoren *et al.* 1985; Dorrington *et al.* 1987; Hutchinson *et al.* 1988), accompanied by an increase in P450arom gene expression (Steinkampf *et al.* 1988). These effects on steroidogenesis are associated with a stimulation of LDL metabolism and receptor expression (Veldhuis *et al.* 1987; Veldhuis & Rodgers, 1987). Other correlates of granulosa cell differentiation which are stimulated by IGF-I include LH receptor induction (Maruo *et al.* 1988), inhibin production (Bicsak *et al.* 1986; Zhang *et al.* 1987a; LaPolt *et al.* 1990b; Michel *et al.* 1991b) and proteoglycan secretion (Adashi *et al.* 1986d). These effects appear to be mediated by specific IGF-I receptors since insulin and IGF-II are far less potent than IGF-I (Veldhuis *et al.* 1987; Maruo *et al.* 1988; Veldhuis *et al.* 1986b). IGF-I also has profound effects on thecal steroidogenesis. Both basal and LH-stimulated androgen production are enhanced by IGF-I (Barbieri *et al.* 1983; Hernandez *et al.* 1988; Cara & Rosenfield, 1988; Magoffin *et al.* 1990); indeed the effect of IGF-I on thecal androgen synthesis is greater than that of LH, and the combination of LH and IGF-I stimulates androgen synthesis synergistically (Hillier *et al.* 1991a; Hillier *et al.* 1991b). Thecal LH binding is also enhanced by IGF-I (Cara *et al.* 1990).

The effects of IGF-I in enhancing gonadotrophin-stimulated granulosa cell differentiation appear to be mediated partly at the level of cAMP generation or breakdown, since IGF-I treatment of cultured granulosa cells has been shown to increase accumulation of cAMP (Davoren *et al.* 1985; Adashi *et al.* 1986c). However, the fact that stimulation of cells with maximally effective doses of cAMP analogues can be further augmented by IGF-I (Adashi *et al.* 1986b; Veldhuis & Furlanetto, 1985) suggests that it may also increase cellular responsiveness to cAMP. IGF-I action is mediated through a receptor which has tyrosine kinase activity (Yarden & Ullrich, 1988; Rechler & Nissley, 1985), but the nature of the proteins phosphorylated in response to IGF-I binding is unknown. It is conceivable that the IGF-I receptor

phosphorylates some component or components of the cAMP response and metabolism mechanisms.

Although IGF-I is the major, if not only, IGF produced by rat and pig granulosa cells, theca cells express the IGF-II gene (Hernandez *et al.* 1990b). In humans, IGF-II is found in follicular fluid (Ramasharma *et al.* 1986), and there is evidence that granulosa cells produce IGF-II, and that its expression is stimulated by FSH, LH, GH and agents which stimulate the cAMP second messenger system (Voutilainen & Miller, 1987b; Ramasharma & Li, 1987). It has been confirmed that human granulosa cells express the IGF-II and not the IGF-I gene (Geisthovel *et al.* 1989a), but this does not explain the high concentrations of IGF-I in human follicular fluid (Geisthovel *et al.* 1989b; Rabinovici *et al.* 1990a).

Attempts to understand the function of IGFs within the ovary have been considerably complicated by the expression of IGF-binding proteins (IGF-BPs) in ovarian cells. Unlike insulin, the vast majority of IGFs that are transported in the blood are bound to IGF-BPs, which affect the presentation of IGFs to their receptors, but there are at least six types of IGF-BPs, which may have different modulatory effects on IGF effects (Rosenfeld *et al.* 1990; Shimasaki *et al.* 1991). Human granulosa cells produce IGF-BP-1 mRNA and protein (Jalkanen *et al.* 1989; Koistinen *et al.* 1990; Suikkari *et al.* 1989). Very recently, Nakatani et al (1991b) have studied the expression of four IGF-BPs (types 1,2,3 and 4) in the rat ovary by in situ hybridisation. They found no IGF-BP-1 mRNA anywhere in the ovary, in agreement with studies on pig granulosa cells (Mondschein *et al.* 1990). However, the other three IGF-BPs were found to be expressed in a highly compartmentalised pattern. IGF-BP-2 was expressed only in thecal and interstitial cells, in agreement with Ricciarelli et al (1991), being expressed more in larger follicles. IGF-BP-3 mRNA was found only in corpora lutea, and IGF-BP-4 mRNA was detected only in the granulosa cells of atretic follicles, with the expression being greatest in follicles in more advanced stages of atresia. The authors assumed the IGF-BPs to be inhibitory (with some justification) and were led to suggest that since luteal cells and granulosa cells of atretic follicles are not proliferating, their expression of IGF-BPs might be acting to block the mitogenic actions

of IGFs. Increased granulosa cell IGF-BP production is also associated with atresia in the sheep (Monget *et al.* 1989).

Both stimulatory and inhibitory effects of IGF-BPs on IGF action have been shown (Adashi *et al.* 1991), but to date, the evidence suggests that IGF-BPs inhibit the actions of IGFs on granulosa cells. In fact, the porcine IGF-BP-3 was isolated and cloned on the basis of its ability to inhibit FSH-stimulated oestradiol production by cultured rat granulosa cells (Ui *et al.* 1989; Shimasaki *et al.* 1989a). Two IGF-BPs, types 2 and 3, inhibit granulosa cell steroidogenesis stimulated *in vitro* by FSH or a cAMP analogue (but, paradoxically, not by LH), without any effect on cAMP production, and also inhibit proliferation (Bicsak *et al.* 1990). The effects of the IGF-BPs were very similar to those of a specific antiserum to IGF-I, suggesting that in the rat, IGF-BPs of granulosa or thecal origin are capable of inhibiting granulosa cell function by binding to and neutralising endogenous IGF-I.

6.2.2 EGF and TGFα

Epidermal growth factor (EGF) affects both proliferation and differentiation of granulosa cells in several species. Granulosa cells of rats and pigs have been shown to have specific EGF receptors (Jones *et al.* 1982; Buck & Schomberg, 1988), which are upregulated by treatment with FSH *in vivo* (St Arnaud *et al.* 1983; Siebers *et al.* 1985) and *in vitro* (Feng *et al.* 1986; Feng *et al.* 1987; Buck & Schomberg, 1988). Autoradiographic studies have also localised EGF receptors to thecal, interstitial and luteal cells (Feng *et al.* 1987; Chabot *et al.* 1986). However, EGF does not appear to be expressed in the ovary (Skinner & Coffey, 1988). Another protein, transforming growth factor-α (TGFα), which shares about 40% homology with EGF, and interacts with the same receptor as EGF (Massague, 1985; Carpenter, 1987; Gill *et al.* 1987), has been shown to be expressed in thecal cells (Kudlow *et al.* 1987; Skinner & Coffey, 1988; Lobb *et al.* 1989). It seems likely, therefore, that the observed effects of EGF on granulosa cells reflect the function of TGFα *in vivo*.

EGF is a potent mitogen for granulosa cells of a number of species *in vitro*, including cows, pigs, humans and rabbits (Gospodarowicz &

Bialecki, 1979; Mondschein *et al.* 1988; May *et al.* 1987; Tapanainen *et al.* 1987; Bendell *et al.* 1988; Skinner *et al.* 1987), although rat granulosa cells, on which the effects of EGF have been studied most intensively, are relatively insensitive to the mitogenic action of EGF (Zhang *et al.* 1987b; Gospodarowicz & Bialecki, 1979). The stimulation of proliferation by EGF depends upon the culture system and the presence of other factors (Mondschein & Hammond, 1988; May *et al.* 1990). As one would expect of a factor which promotes proliferation, EGF generally inhibits granulosa cell differentiation. In rats, EGF inhibits FSH-stimulated production of oestradiol (Dorrington *et al.* 1987; Jones *et al.* 1982; Adashi *et al.* 1987; Suzuki *et al.* 1987), inhibin (Bicsak *et al.* 1986; Zhang *et al.* 1987b; Zhang *et al.* 1988a) and progesterone (Suzuki *et al.* 1987; Dodson & Schomberg, 1987) and LH receptor induction (Feng *et al.* 1986; Dodson & Schomberg, 1987). These effects are apparently due to a suppression of cAMP production (Adashi *et al.* 1987; Dodson & Schomberg, 1987; Ben Ze'ev & Amsterdam, 1987), although it is also able to inhibit the action of cAMP analogues (Adashi *et al.* 1987; Zhang *et al.* 1987b). The effects of EGF are similar in pigs, cows and humans (Steinkampf *et al.* 1988; Franchimont *et al.* 1986; Mason *et al.* 1990; Michel *et al.* 1991b), except that progesterone production by pig and human granulosa cells may be enhanced by EGF (Tapanainen *et al.* 1987; Richardson *et al.* 1989; Urban *et al.* 1990). Also, in pigs, EGF increases FSH receptor numbers (May *et al.* 1987) and stimulates IGF-I and IGF-BP production (Mondschein *et al.* 1988; Mondschein & Hammond, 1988; Mondschein *et al.* 1990). To date, the effects of TGFα have been shown to be the same as those of EGF (Adashi & Resnick, 1986; Skinner & Coffey, 1988; Tsafriri *et al.* 1989b).

6.2.3 The TGFβ Family

As the name suggests, transforming growth factor (TGF) was originally described on the basis of its ability reversibly to elicit transformation of normal cells, similar to viral transformation, which is characterised by cell proliferation uninhibited by cell contact and independent of cell attachment (Massague, 1985). Upon fractionation, TGF was found to consist of two components, one related to EGF and

termed TGFα, and another which seemed to synergise with TGFα in inducing transformation, termed TGFβ. TGFβ has now been found to exist in three forms, $TGF\beta_1$, $TGF\beta_2$, and $TGF\beta_3$, which are all homodimers of ~12kDa subunits, linked by disulphide bridges. Furthermore, there are also a number of proteins which are related to TGFβ. These proteins share a number of structural features, such as their distribution of cysteine residues involved in proteolytic processing of precursors, and the fact that they are all active as homodimers. Considerable impetus has been added to the study of inhibin and activin by the recent discovery that they are members of this family (Mason *et al.* 1985; Derynck & Rhee, 1987). TGFβ, inhibins and activins also share significant homology with Mullerian duct Inhibiting Substance (MIS) (Cate *et al.* 1986), which causes regression of the Mullerian duct during the early embryonic differentiation of the testis, and a product of the *Drosophila* decapentaplegic complex (Dpp), which is also involved in early embryonic development (Padgett *et al.* 1987). With the exception of the *Drosophila* protein, all these members of the TGFβ family are expressed within the ovary, including MIS (Takahashi *et al.* 1986; Voutilainen & Miller, 1987a). All of these factors are clearly biologically very important molecules, and comparison of their actions may provide valuable insight into the biology of inhibin and activin and *vice versa*.

The regulation of ovarian TGFβ expression has not been studied in detail, but it is clear that theca cells of rats, pigs and cows at least produce TGFβ (Bendell & Dorrington, 1991; Skinner *et al.* 1987; Bendell & Dorrington, 1988; Kim & Schomberg, 1989; Gangrade & May, 1990; Hernandez *et al.* 1990a). Pig granulosa cells do not produce TGFβ (Gangrade & May, 1990; Kim & Schomberg, 1989) but rat granulosa cells do, at least *in vitro* (Kim & Schomberg, 1989; Bendell & Dorrington, 1991), and its production is stimulated by oestradiol (Bendell & Dorrington, 1991). In contrast to its expression, the effects of TGFβ on ovarian cells, especially granulosa cells, have been extensively studied. There is considerable variation between the responses of rat and pig granulosa cells to TGFβ. In rats, TGFβ augments several effects of FSH, including production of oestradiol (Hutchinson *et al.* 1987; Ying *et al.* 1986; Adashi & Resnick, 1986; Bendell & Dorrington, 1988; Adashi *et al.* 1989) inhibin

(LaPolt *et al.* 1989; Zhang *et al.* 1988a; LaPolt *et al.* 1990b) and progesterone (Dodson & Schomberg, 1987; Knecht *et al.* 1987) and also LH and EGF receptor induction (Blair *et al.* 1988; Feng *et al.* 1986). These effects are partly due to enhanced cAMP accumulation (Knecht *et al.* 1986; Blair *et al.* 1988), but also appear to increase responsiveness to cAMP or cAMP analogues (Knecht *et al.* 1987; Feng *et al.* 1986; Adashi *et al.* 1989). They also appear to be specific, since antibodies to TGFβ have been used to block its effects (Bendell & Dorrington, 1988; Blair *et al.* 1988). However, TGFβ inhibition of granulosa cell cAMP production and differentiation stimulated by a high dose of FSH has also been observed in rat cells, but only in the presence of insulin (Knecht *et al.* 1986; Knecht *et al.* 1987) or EGF (Zhang *et al.* 1988a). In rats TGFβ also stimulates proliferation of granulosa cells, both alone (Dorrington *et al.* 1988) and in synergy with FSH (Dorrington *et al.* 1988; Bendell & Dorrington, 1988), and the action of FSH in stimulating proliferation (where it has been observed) can be blocked with an antibody to TGFβ (Bendell & Dorrington, 1991). Whereas in rats TGFβ is generally a stimulatory factor, in pigs it is the opposite. TGFβ inhibits FSH-stimulated porcine granulosa cell production of inhibin (Michel *et al.* 1991b) and progesterone (Mondschein *et al.* 1988) as well as proliferation (Mondschein *et al.* 1988; Gangrade & May, 1990). It also inhibits EGF-stimulated IGF-I production (Mondschein *et al.* 1988; Mondschein & Hammond, 1988) and basal IGF-BP secretion (Mondschein *et al.* 1990), but enhances EGF-stimulated proliferation (May *et al.* 1988). In the cow it also inhibits EGF-stimulated granulosa cell proliferation (Skinner *et al.* 1987).

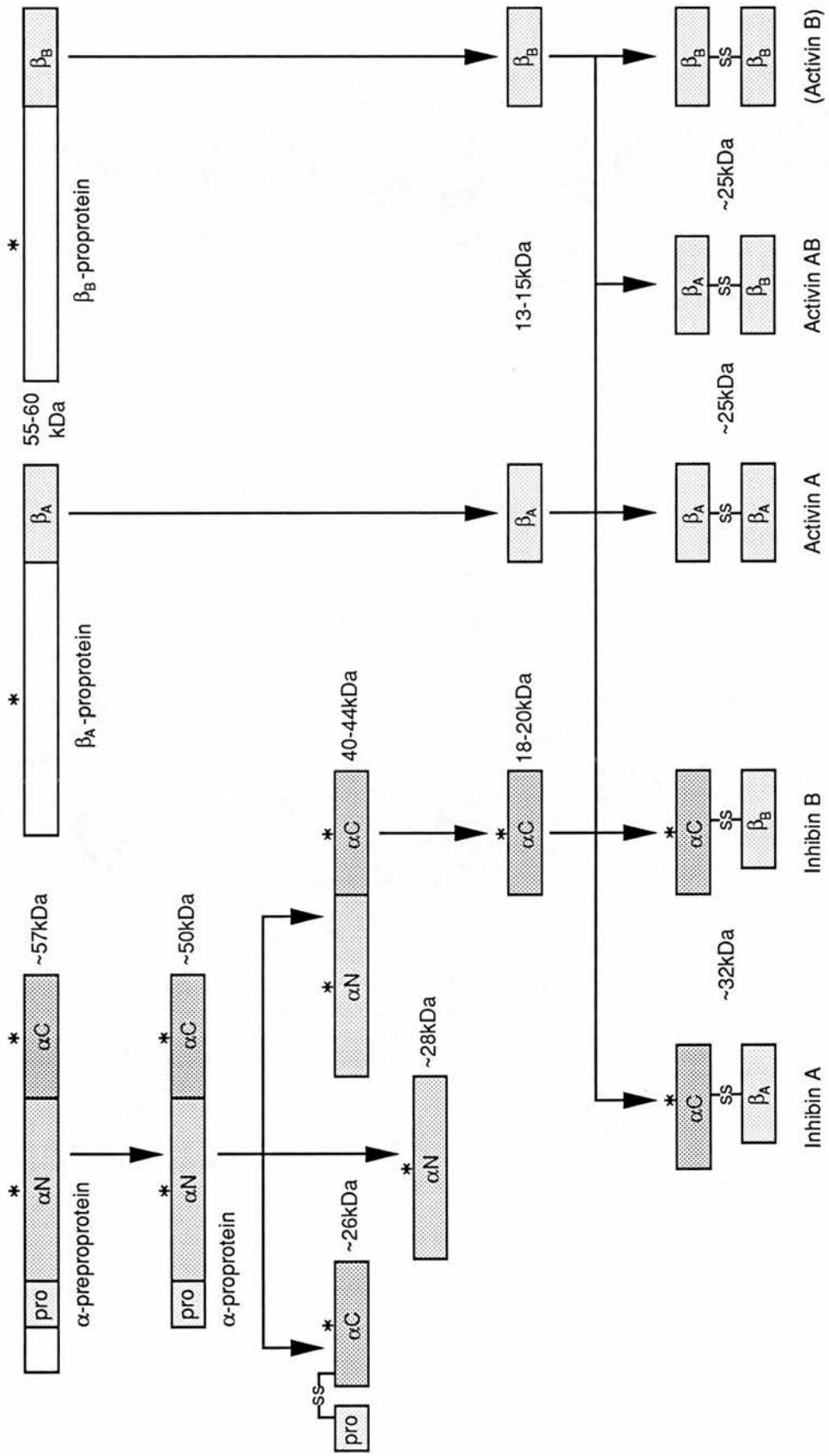
Data on the effects of TGFβ on thecal cells are more clear, perhaps because they are less abundant. In the rat, TGFβ appears to stimulate thecal progesterone production, while inhibiting androgen production without affecting proliferation (Magoffin *et al.* 1989; Hernandez *et al.* 1990a). These effects were seen only in the presence of insulin or IGF-I. In cow theca cells, TGFβ inhibits proliferation, has no effect on androgen production, and stimulates progesterone production (Roberts & Skinner, 1991).

6.2.4 Inhibins and Activins

Inhibin is a heterodimeric glycoprotein consisting of an α-subunit and one of two closely related β-subunits, termed β_A and β_B. This protein was purified from follicular fluids from a number of species, on the basis of its ability to suppress FSH release from pituitary cells (Burger & Igarashi, 1988) (see above). The smallest form of inhibin purified from follicular fluid is a protein with a molecular weight of about 32kDa, consisting of an α-subunit of 18-20kDa and a β-subunit of 13-15kDa (Leversha *et al.* 1987; Mason *et al.* 1985; Rivier *et al.* 1985; Miyamoto *et al.* 1985). Inhibin A denotes a dimer of an α- and a β_A-subunit, whereas inhibin B is a dimer of an α- and a β_B-subunit. Both types have been purified from porcine follicular fluid (Ling *et al.* 1985), and testicular inhibin appears to be very similar (Vaughan *et al.* 1989; Grootenhuis *et al.* 1990).

However these are not the only combinations of inhibin subunits which are produced by the gonads (Miyamoto *et al.* 1986). Some of the forms which have been identified in follicular fluid are shown in Fig.1.4. The α-subunit is synthesized as a 360 amino acid precursor, which is processed in a number of proteolytic steps to form mature α-subunit, or αC. Amino acids 18-60 comprise the "pro-" sequence, amino acids 61-226 the αN sequence, and amino acids 227-360 make up the mature αC. A 58kDa form of inhibin has been found in bovine follicular fluid, the α-subunit of which was about 44kDa in size (Robertson *et al.* 1985; Forage *et al.* 1986), corresponding to the uncleaved αN-αC molecule. In addition, a 26kDa α-subunit has been purified from bovine follicular fluid which is composed of the αC fragment, linked by disulphide bond(s) to the pro-peptide (Sugino *et al.* 1989), and is known as pro-αC. This molecule was also found by Robertson *et al.* (1989), who also found the intervening αN cleaved peptide. There is also evidence that free α- (Bicsak *et al.* 1988; Knight *et al.* 1989; Schneyer *et al.* 1990) and β- (Robertson *et al.* 1992) subunit monomers are secreted by ovarian cells, although the significance of this is not known. Much higher molecular weight forms of inhibin have also been identified by Western blotting, which appear to be composed of a large (~62kDa) β-subunit precursor, linked to αβ dimers of different sizes

Fig.1.4. Some forms of inhibins and activins found in the follicular fluid of several species. Consensus figures for the molecular weights of subunits and dimers are shown in kilodaltons (kDa). SS indicates that subunits are connected by disulphide bonds, and an asterisk indicates that a subunit is glycosylated. Note that activin B has not been identified in follicular fluid.


pro
αN
αC
~57kDa
α-prepreprotein
~50kDa
α-proprotein
~26kDa
~28kDa
40-44kDa
18-20kDa
αC
αN
pro
ss
βA
βB
βA-proprotein
βB-proprotein
55-60 kDa
13-15kDa
~25kDa
~32kDa
ss
Inhibin A
Inhibin B
Activin A
Activin AB
(Activin B)

(Miyamoto *et al.* 1986). Very recently, Sugino *et al.* (1992) reported the purification from bovine follicular fluid of several more high molecular weight forms of inhibin, which appear to be composed of a β-subunit either 15kDa or 55kDa in size and an α-subunit of either 40kDa or 50kDa.

As well as peaks of protein which inhibited release of FSH from pituitary cells, Ling *et al.* (1985) found that some porcine follicular fluid fractions eluting from a column before inhibin contained an activity which was capable of *stimulating* release of FSH. This activity was subsequently found to be a dimer of two inhibin β-subunits, one β_A and one β_B (Ling *et al.* 1986b). Homodimers of β_A-subunits have also been purified from porcine follicular fluid, having the same properties as β_A-β_B dimers (Vale *et al.* 1986; Ling *et al.* 1986a). These proteins, termed activin A (β_A-β_A) and activin AB (β_A-β_B) have now been found to affect a wide range of tissues, and where studied, their effects have always been shown to be the opposite to those of inhibin.

Detection methods for the study of the hormonal regulation of inhibin production originally relied on cultured pituitary cell bioassay, by measuring FSH production (Erickson & Hsueh, 1978; Bicsak *et al.* 1986), or radioimmunoassays (RIA), using antibodies raised against synthetic peptide fragments of the α-subunit (Rivier *et al.* 1987; Rivier *et al.* 1986). Antibodies have also been used to study the distribution of inhibin subunits by immunocytochemistry (Merchenthaler *et al.* 1987; Meunier *et al.* 1988a). Data obtained by RIA are difficult to interpret, due to the wide variety of possible combinations of inhibin subunits and subunit fragments. Using oligonucleotides designed on the basis of partial protein sequence data, cDNA encoding all three inhibin subunits has now been cloned from rat ovary (Woodruff *et al.* 1987; Hsu & Hammond, 1987a), pig ovary (Mason *et al.* 1985; Mayo *et al.* 1986), human ovary, testis and placenta (Mason *et al.* 1986; Mason *et al.* 1989; Keinan *et al.* 1989; Feng *et al.* 1989a; Mayo *et al.* 1986), and cow granulosa cell (Forage *et al.* 1986) cDNA. These cDNA fragments have been used to study the regulation of expression of inhibin subunits at the level of their mRNA. The inhibin genes have also been cloned in the rat (Feng *et al.* 1989b; Albiston *et al.* 1990), sheep (Rodgers, 1991) and human (Albiston *et al.* 1990) and have one intron each, situated in the region coding for the precursor

sequences. The α- and β_B- genes have been mapped to human chromosome 2 and mouse chromosome 1 and the β_A- gene to human chromosome 7 and mouse chromosome 13 (Barton *et al.* 1989; Chenevix Trench *et al.* 1990). The three subunit genes bear considerable homology with one another, and are highly conserved between species (in fact, the human, rat, cow and pig β_A-subunit proteins are identical), suggesting that they all evolved from a single ancestral gene.

Using various assays, it has been shown that the principal gonadal sites of inhibin expression are the granulosa cells in the ovary and the Sertoli cells in the testis. It has been shown by immunocytochemistry that granulosa cells produce all three inhibin subunits (Meunier *et al.* 1988a), and in situ hybridisation experiments have shown that the levels of mRNA encoding each subunit correlate with changes in protein expression (Meunier *et al.* 1988a; Rivier *et al.* 1989; Woodruff *et al.* 1989). In rats, cows and sheep, the α-subunit is expressed in healthy follicles of all sizes, but the β-subunits are only expressed in healthy antral follicles, and not in atretic or preovulatory follicles (Rodgers *et al.* 1989; Woodruff *et al.* 1988; Meunier *et al.* 1988a). In the primate, there is a recent report that the β_B-subunit is expressed at an earlier stage of follicular development than the α- or β_A-subunits (Schwall *et al.* 1990). In the case of species such as rats, cows and sheep, the corpus luteum does not express inhibin (Mann *et al.* 1989b; Campbell *et al.* 1991; Torney *et al.* 1989; Rodgers *et al.* 1989; Woodruff *et al.* 1988; Woodruff *et al.* 1989), although some inhibin α-subunit and α-subunit mRNA has been detected in newly formed rat corpus luteum (Cuevas *et al.* 1987; Meunier *et al.* 1988a). In contrast, human and other primate corpora lutea do produce bioactive inhibin and express inhibin subunits (Tsonis *et al.* 1987; Davis *et al.* 1987; Hillier *et al.* 1989b; Smith & Fraser, 1991). In rat and cow theca interna cells, there is evidence that there is some expression of the inhibin α-subunit, although only at low levels (Meunier *et al.* 1988a; Rivier *et al.* 1989; Torney *et al.* 1989).

The expression of the inhibin genes in the ovary is under the control of gonadotrophins, and varies during the ovarian cycle in response to changes in levels of gonadotrophins. In humans and other primates, serum inhibin levels follow roughly the same pattern as

oestradiol. Levels of inhibin are low during the early follicular phase, and rise during the late follicular phase to reach a peak just prior to the midcycle gonadotrophin surge, when inhibin levels drop (McLachlan *et al.* 1987; McLachlan *et al.* 1990; Reddi *et al.* 1990). When the corpus luteum is established, inhibin levels rise again. With the regression of the corpus luteum, inhibin levels fall along with those of progesterone and oestradiol (McLachlan *et al.* 1987; Roseff *et al.* 1989; Smith *et al.* 1990c), and this can be experimentally reproduced by luteectomy (Bassett *et al.* 1990) or infusion of a GnRH antagonist during the luteal phase (Fraser *et al.* 1989). Similarly, inhibin levels can be maintained by "rescue" of the corpus luteum with hCG in humans (Illingworth *et al.* 1990). The peak of inhibin just before the surge is not so pronounced as, and occurs later than, that of oestradiol. While oestradiol levels during the luteal phase are elevated in these species, they never reach the levels seen just before midcycle. However, inhibin levels in the blood are at their highest during the luteal phase. Secretion of inhibin during the primate ovarian cycle can be said to follow a pattern somewhere between that of oestradiol and that of progesterone. In species such as rats, sheep and cows, the pattern of inhibin secretion and gene expression follows a different pattern. In rats, serum and follicular fluid inhibin levels begin to rise after the secondary FSH surge in the morning of oestrus (Watanabe *et al.* 1990; Hasegawa *et al.* 1989; Fujii *et al.* 1983). This can be mimicked by unilateral ovariectomy, which causes an increase in serum FSH levels, which leads to recruitment of more follicles, and increased expression of inhibin subunit mRNA in these follicles (D'Agostino *et al.* 1989; Ackland *et al.* 1990). Inhibin levels increase steadily during dioestrus and metoestrus, to reach a peak just before the gonadotrophin surge in the afternoon of prooestrus. Thereafter, serum inhibin levels (Yohkaichiya *et al.* 1991; Rivier *et al.* 1989; Watanabe *et al.* 1990) and expression of inhibin subunit genes in preovulatory follicles (Meunier *et al.* 1988a; Woodruff *et al.* 1988) decline sharply, although in smaller follicles inhibin expression remains essentially unchanged. This fall in inhibin production can be shown to be a response to the surge of LH, since treatment of animals with GnRH antagonists blocks the drop in inhibin expression in the granulosa cells of preovulatory follicles (Rivier *et al.* 1989; Woodruff *et al.* 1989). As with

rats, sheep and cow follicles but not corpora lutea produce inhibin (Campbell *et al.* 1991; Mann *et al.* 1989b), reflecting expression of inhibin α- and β_A-subunit mRNA (Rodgers *et al.* 1989). In these species, inhibin expression is not confined to the largest follicles, and so does not entirely follow the pattern of oestradiol secretion.

In vitro studies have now established that the production of inhibin by rat (Bicsak *et al.* 1986; Suzuki *et al.* 1987; Zhang *et al.* 1987b; Bicsak *et al.* 1988), pig (Michel *et al.* 1991b) and primate (Hillier *et al.* 1989b) granulosa cells is stimulated by FSH. Sheep and cow granulosa cell inhibin production appears to be insensitive to FSH and LH (Henderson & Franchimont, 1981; Campbell *et al.* 1991), although this could be due to the degree of maturity of the granulosa cells or the culture system used. LH has no effect on inhibin production by immature granulosa cells (Zhang *et al.* 1988b; Hillier *et al.* 1989b), but it has a biphasic effect on inhibin production by granulosa cells which have been exposed to FSH, being stimulatory at low doses (Bicsak *et al.* 1986; Hillier *et al.* 1989b) and inhibitory at high concentrations (Zhang *et al.* 1988b). Other factors also affect granulosa cell inhibin production *in vitro,* including steroids (as discussed above) and growth factors (see below). The control of inhibin expression had not been fully investigated at the outset of this project, and the effects of gonadotrophins and steroids on the expression of the inhibin genes in rat granulosa cells are presented in Chapter 4.

Although inhibin and activin were originally postulated as regulators of gonadotrophin secretion, and were purified on the basis of these properties, it is becoming increasingly clear that these proteins, like steroids, are not only produced in the ovary, but are also capable of acting as paracrine or autocrine regulators of ovarian function. Few *in vivo* studies have examined the effects of inhibin and activin at the level of the ovary. Chari *et al.* (1985) reported that an inhibin-like component of human follicular fluid (based on its ability to prevent the rise in serum FSH in adult rats following ovariectomy) also had the property of inhibiting gonadotrophin-stimulated oestradiol secretion when injected into immature rats. Also, a study has been published recently, in which inhibin or activin were injected into the space between the ovary and its bursa in intact immature rats (Woodruff *et al.* 1990). The results were

slightly confused by the effects of these treatments on gonadotrophin secretion, but the effects observed were that inhibin stimulated follicle development and uptake of tritiated thymidine into granulosa cells, whereas activin caused atresia, decreased granulosa cell tritiated thymidine uptake and partially antagonized the stimulatory effect of PMSG on follicle development. Specific binding of both inhibin and activin to granulosa cells was also demonstrated.

In rat granulosa cell cultures, inhibin appears to have little effect, either slightly inhibiting FSH-stimulated cAMP generation, leading to a reduction in aromatase activity and progesterone production (Ying *et al.* 1986), or having no effect at all (Hutchinson *et al.* 1987). Activin, in contrast, has been convincingly shown to stimulate granulosa cell function, augmenting the stimulation of cAMP formation (Xiao & Findlay, 1991), LH receptor expression (Sugino *et al.* 1988a), aromatase activity (Hutchinson *et al.* 1987; Xiao *et al.* 1990), progesterone production (Sugino *et al.* 1988a; Xiao *et al.* 1990; Xiao & Findlay, 1991) and inhibin gene expression (LaPolt *et al.* 1989) and production (Sugino *et al.* 1988a; LaPolt *et al.* 1989; Xiao *et al.* 1990; Xiao & Findlay, 1991) brought about by FSH or forskolin. Activin also increases the numbers of FSH receptors on granulosa cells (Hasegawa *et al.* 1988). Some of the stimulatory effects of activin on granulosa cells are mimicked by TGFβ (Adashi & Resnick, 1986; Ying *et al.* 1986; Dodson & Schomberg, 1987; Hutchinson *et al.* 1987), which is structurally related to activin (see above), but high affinity binding sites for activin which have been demonstrated on cultured rat granulosa cells (Sugino *et al.* 1988b; LaPolt *et al.* 1989; Woodruff *et al.* 1990) do not bind TGFβ (Sugino *et al.* 1988b). Activin has also been shown to stimulate proliferation of luteinizing human granulosa cells (Rabinovici *et al.* 1990b) and to inhibit progesterone and oxytocin production (both markers of luteinized function) by luteinizing cow granulosa cells (Shukovski & Findlay, 1990), suggesting that while it promotes early granulosa cell development, activin inhibits luteinization.

Both inhibin and activin also modulate thecal cell androgen production. Hsueh *et al.* (1987) showed that inhibin markedly augments LH-stimulated androgen production by cultured rat thecal cells, and that

the effects of activin were quite the opposite. This has recently been shown to be the case for human thecal cells also (Hillier *et al.* 1991a; Hillier *et al.* 1991b), both factors selectively regulating androgen production, and only affecting progesterone production at high doses, and without affecting cell division.

It has also suggested that inhibin acts as an inhibitor of oocyte maturation (Tsafriri *et al.* 1989b; O *et al.* 1989), and α-subunit fragments may also facilitate ovulation (Findlay *et al.* 1990).

Although the gonads are the richest source of inhibin, the expression of inhibin and related proteins is not limited to these tissues. Using the highly sensitive and specific technique of S1 nuclease protection assay, Meunier *et al.* (1988b) demonstrated expression of inhibin subunit mRNA in a wide variety of non-gonadal rat tissues, including placenta, pituitary, adrenal, bone marrow, spleen, kidney, brain and spinal cord. Expression of inhibin subunits in the adrenal cortex has been confirmed, and found to be under the control of ACTH and corticosteroids (Crawford *et al.* 1987; Haji *et al.* 1991). Likewise, placental inhibin expression has been confirmed in the Japanese monkey (Nozaki *et al.* 1990) and human (McLachlan *et al.* 1986; Petraglia *et al.* 1987), where it is stimulated by hCG and cAMP analogues, and may be involved in the local control of hCG secretion (Petraglia *et al.* 1987). Inhibin production by the placenta in other species is still uncertain, since another study failed to find mRNA encoding inhibin-α in the rat placenta (Davis *et al.* 1986c). Unexpected sources of inhibin gene expression are the pituitary gonadotroph cells, where α- and β_B-subunit mRNA and protein have been detected, and found to be regulated by oestradiol (Roberts *et al.* 1989). Furthermore, an antibody to activin-B suppresses basal FSH secretion in cultured rat pituitary cells (Corrigan *et al.* 1991), suggesting that locally produced activin is an autocrine regulator of FSH secretion. Activin-A has also been detected using a specific radioimmunoassay in the rat brain and human placenta, as well as the gonads and pituitary, although by far the highest levels of activin detected were found in the gonads (Shintani *et al.* 1991). A factor known as erythroid differentiation factor (EDF), which induces the differentiation of erythrocytes from stem cells, has been found to be identical to activin A (Eto *et al.* 1987; Murata *et al.* 1988).

This has been confirmed in studies which showed that recombinant activin A has the same effects as EDF on blood cells (Yamashita *et al.* 1990; Broxmeyer *et al.* 1988) and on the differentiation of nerve cells (Hashimoto *et al.* 1990). Activins are also expressed during early embryonic development at least in *Xenopus* (Thomsen *et al.* 1990) and chicks (Mitrani *et al.* 1990), where they induce tissue differentiation (van den Eijnden-Van Raaij *et al.* 1990; Smith *et al.* 1990b).

6.2.5 Follistatin

Another protein called follistatin, which like inhibin suppresses FSH release from pituitary cells, has been purified from pig and cow follicular fluid. This protein is a single-chain polypeptide sharing no homology with inhibin, which exists in two forms with molecular weights of 32kDa and 35kDa in pig follicular fluid (Ueno *et al.* 1987), and three forms with molecular weights of 31kDa, 35kDa and 39kDa in cow follicular fluid (Robertson *et al.* 1987). These proteins, without affecting secretion of LH, suppress FSH secretion stimulated by GnRH or activin *in vivo* (DePaolo *et al.* 1991) and *in vitro* (Ueno *et al.* 1987; Robertson *et al.* 1987), although with lower potency than inhibin (Ying *et al.* 1987; Robertson *et al.* 1990; DePaolo *et al.* 1991). This effect is partly exerted by reducing levels of FSH-β mRNA (Carroll *et al.* 1989), and partly by inhibiting FSH release (Ying *et al.* 1987). The effects of inhibin and follistatin on FSH secretion are parallel, additive and equal at maximally effective doses (Ying *et al.* 1987), suggesting a common mechanism of action. Following the cloning of cDNA and the genes (which consist of six exons) encoding pig and rat follistatin, it was established that the size variants are the result of alternate splicing, and not products of different genes (Shimasaki *et al.* 1988; Michel *et al.* 1990; Shimasaki *et al.* 1989b). In pig follicular fluid, the 35kDa protein is by far the more abundant (Ueno *et al.* 1987; Michel *et al.* 1990), and it has been shown that the larger the form of bovine follistatin, the more potent its suppression of FSH secretion (Wang *et al.* 1990). However, a recent paper reported that of the two forms of recombinant human follistatin generated by the authors, it

was the smaller which was very much the more active, being as effective at inhibiting FSH secretion as inhibin (Inouye *et al.* 1991).

An interesting property of follistatin is its ability to bind activin (Nakamura *et al.* 1990), which may explain its ability to antagonize the effects of activin in a large number of studies (Ying, 1989; Carroll *et al.* 1989; Xiao *et al.* 1990; Xiao & Findlay, 1991). Follistatin also suppresses GnRH-stimulated FSH secretion in the absence of activin, which cannot be explained by this property, unless the action of GnRH involves locally produced activin (see above), which seems unlikely considering how quickly LH secretion follows a pulse of GnRH (Lincoln *et al.* 1985).

Expression of follistatin and its mRNA in the rat ovary follows a similar pattern to that of inhibin-α. Expression of follistatin mRNA is confined to the granulosa cells of growing follicles from the early antral stage onwards (Shimasaki *et al.* 1989b; Nakatani *et al.* 1991a). Follistatin mRNA and protein levels increase with the size of the follicle, to a maximum in the preovulatory follicle before the LH surge, after which expression falls, lingering in the newly-formed corpus luteum but absent by the time of luteal regression (Nakatani *et al.* 1991a). *In vitro* and *in vivo*, follistatin production by granulosa cells is stimulated by FSH or PMSG (Michel *et al.* 1990; Shimasaki *et al.* 1989b; Saito *et al.* 1991).

As with the other factors discussed above, expression of follistatin is not limited to the ovary. The discovery of follistatin in the pituitary itself (Kogawa *et al.* 1991; Michel *et al.* 1990) adds yet another layer of complexity to the control of FSH secretion. Is FSH secretion modulated by locally produced activin? If so, is that also subject to modulation by locally produced follistatin? A study using the highly sensitive technique of S1 nuclease protection assay demonstrated follistatin expression in rat brain, adrenal, thymus, pancreas, gut, kidney, heart, uterus, skeletal muscle and lung as well as ovary, testis and pituitary. Another study confirmed by Northern blotting the expression of follistatin in the ovary, kidney and brain, but failed to detect the signal in other tissues (Shimasaki *et al.* 1989b). Follistatin is also expressed at the same time as activin during development of *Xenopus* embryos (Tashiro *et al.* 1991).

There is little information available at the moment on the effects of follistatin on ovarian cells. Follistatin appears to have no effect on

progesterone secretion by cultured preovulatory follicles, or on the maturation of their oocytes (Tsafriri *et al.* 1989b). However, two recent studies have shown inhibitory effects of follistatin on the FSH-stimulated secretion of inhibin and oestradiol by cultured rat granulosa cells (Xiao *et al.* 1990), due to a suppression of cAMP accumulation (Xiao & Findlay, 1991).

6.2.6 GnRH

The hypothalamic decapeptide gonadotrophin-releasing hormone (GnRH), in addition to its primary function of stimulating pituitary FSH and LH release, has also unexpectedly been found to have direct actions on granulosa cells, which have been studied in some detail. GnRH has been detected in sheep and cow ovaries (Aten *et al.* 1987), and its mRNA has been detected in rat ovary by the extremely sensitive technique of reverse transcriptase-PCR (Oikawa *et al.* 1990). Furthermore, receptors for GnRH have also been demonstrated on rat granulosa and theca cells (Maruo *et al.* 1985; Hsueh & Schaeffer, 1985), and on the granulosa cells of dominant human follicles (Latouche *et al.* 1989).

Treatment of immature granulosa cells with GnRH or a GnRH agonist stimulates fibronectin secretion (Dorrington & Skinner, 1986), while inhibiting FSH-stimulated progesterone (Knecht *et al.* 1982; Wang & Leung, 1986; Suzuki *et al.* 1987; Wang *et al.* 1989b), oestradiol (Hsueh & Schaeffer, 1985; Wang & Leung, 1986; Hillier *et al.* 1987; Suzuki *et al.* 1987) and inhibin (Bicsak *et al.* 1986; Suzuki *et al.* 1987; LaPolt *et al.* 1990b) production, and also FSH-stimulated LH receptor induction (Shinohara *et al.* 1985a; Maruo *et al.* 1985; Knecht *et al.* 1982). In more mature cells, GnRH or GnRH agonist stimulates progesterone (Kawai & Clark, 1985; Eckstein *et al.* 1986; Wang & Leung, 1988; Wang & Leung, 1987b; plasminogen activator (Wang & Leung, 1986; Ny *et al.* 1987; Ohlsson *et al.* 1988) and prostaglandin (Kawai & Clark, 1985; Kawai & Clark, 1986; Wang *et al.* 1989b) production. Many of these effects can be blocked with specific GnRH antagonists (Wang & Leung, 1986; Bicsak *et al.* 1986; Ny *et al.* 1987; LaPolt *et al.* 1990b), suggesting a specific effect of GnRH, mediated via specific receptors. The main reason why GnRH has attracted so much

attention as an intraovarian regulatory peptide when very little is known about the regulation of its production and reception in the ovary is that it inhibits differentiation of granulosa cells through the protein kinase C pathway. GnRH causes a rapid increase in granulosa cell inositol trisphosphate (Davis *et al.* 1986b; Wang & Leung, 1988), diacylglycerol (Davis *et al.* 1986b) and Ca^{2+} (Davis *et al.* 1986b; Wang *et al.* 1989a) levels. Calcium chelating agents, protein kinase C inhibitors and calcium channel blockers inhibit the effects of GnRH (Eckstein *et al.* 1986), and phorbol esters mimic its effects (Shinohara *et al.* 1985a; Kawai & Clark, 1985; Kawai & Clark, 1986; Wang *et al.* 1989b). The action of GnRH on granulosa cells, like its action on pituitary cells (Roberts *et al.* 1988), is therefore very probably mediated through the protein kinase C pathway. The protein kinase A and C pathways are apparently not entirely independent of one another, since GnRH also affects cAMP metabolism by reducing gonadotrophin-stimulated adenylate cyclase activity and stimulating phosphodiesterase activity (Knecht *et al.* 1983a; Knecht *et al.* 1983b), and also reducing FSH receptor numbers (Knecht *et al.* 1983b), thereby reducing the effectiveness of FSH in stimulating generation of cAMP. GnRH and phorbol esters are also capable of inhibiting the granulosa cell response to cAMP (Shinohara *et al.* 1985a; Eckstein *et al.* 1986), further reducing granulosa cell FSH sensitivity.

The function of GnRH in the local control of follicle development is still unknown, but interesting insights into the relative importance of second messenger systems operating in granulosa cells have been gained from its study. The protein kinase A and C signalling systems appear to be absolutely antagonistic in immature cells, differentiation being stimulated through the protein kinase A system and inhibited through the protein kinase C system (Shinohara *et al.* 1985a; Shinohara *et al.* 1985b; Petraglia *et al.* 1987). However, in more mature cells, activation of the protein kinase C system stimulates plasminogen activator secretion (Ny *et al.* 1987) and production of progesterone (Wang & Leung,1987b; Shinohara *et al.* 1986) and prostaglandins (Kawai & Clark, 1985; Wang & Leung, 1989), which suggests a stimulatory role for protein kinase C in ovulation and luteinisation. This concept is not consistent with reports that phorbol esters can stimulate plasminogen activator expression in

immature cells (Ohlsson *et al.* 1988), and inhibit progesterone production by mature cells (Leung *et al.* 1988), nor with a study which found no difference between the responses of immature and mature cells to manipulation of this signalling pathway (Carnegie & Tsang, 1984), and so the physiological importance of the protein kinase C signalling pathway in granulosa cell function remains unknown.

6.2.7 FGFs

Fibroblast growth factors (FGFs) are single chain polypeptides which are mitogenic for many cell types. The name FGF was first given to a pituitary-derived mitogen, based on its ability to stimulate fibroblast growth. FGF was subsequently found to consist of two separate but closely related factors (Baird *et al.* 1986). The pituitary factor was designated basic FGF (bFGF), and its counterpart acidic FGF (aFGF). Since then, growth factors isolated from many different tissues have been found to be either bFGF, aFGF or closely related to them. All these factors share the property of binding to the sulphated glycosaminoglycan heparin, and for this reason these factors are also known as heparin-binding growth factors (Burgess & Maciag, 1989). FGFs, like the members of the TGFβ family, are involved in embryonic development at least in frogs and birds (Cooke & Wong, 1991). Like TGFβ and activin, FGFs cause differentiation of embryonic cells destined to be epidermis cells into mesoderm-type cells (Smith *et al.* 1990b); TGFβ-like factors induce dorsal structures and FGFs induce ventral ones (Slack, 1990). However, the property for which FGFs have attracted the most interest from the ovarian point of view is their potent angiogenic activity (i.e. their ability to stimulate the proliferation of capillary endothelial cells to form new blood vessels). In fact, bioassays for FGF have been used, based on the stimulation of vascularisation of transplanted chick chorioallantoic membranes, rabbit cornea and hamster cheek pouch (Gospodarowicz & Ferrara, 1989). As discussed above, blood vessels invade the collapsed follicle after ovulation, and this is associated with local production of an angiogenic factor, which was subsequently identified as bFGF (Baird *et al.* 1986). bFGF mRNA has been detected in cow corpora lutea, and its production in cultured cow luteal cells is

stimulated by LH (Stirling *et al.* 1991). In this study, bFGF mRNA was not detected in follicles, but bFGF has been detected by immunocytochemistry in granulosa cells of cow follicles of all stages of development (Grothe & Unsicker, 1989). Moreover, bFGF mRNA has been detected in cultured bovine granulosa cells which also secreted bFGF protein into the medium (Neufeld *et al.* 1987). Expression of mRNA encoding both bFGF and aFGF has been detected in rat ovaries, using reverse transcriptase-PCR (Koos & Olson, 1989; Koos & Seidel, 1989). It was found that all of the bFGF and most of the aFGF expression occurs in cells other than the granulosa cells. The authors favoured ovarian endothelial cells as the major site of FGF expression. This difference between the rat and cow is reflected in the fact that FGF stimulates proliferation of cow granulosa cells (Neufeld *et al.* 1987), but is not mitogenic for rat granulosa or theca cells (Gospodarowicz & Bialecki, 1979; Dorrington *et al.* 1988; Adashi *et al.* 1988a; Hurwitz *et al.* 1990). Rat granulosa cells do respond to FGF, however, but the effects are exerted on differentiation rather than growth. bFGF suppresses FSH-stimulated production of oestradiol (Baird & Hsueh, 1986; Adashi *et al.* 1988a) and inhibin (LaPolt *et al.* 1990b), and induction of LH receptors (Oury & Darbon, 1988; Baird & Hsueh, 1986) in rat granulosa cells, and inhibits LH-stimulated androgen production in rat theca cells (Hurwitz *et al.* 1990). Not all its effects are inhibitory, since bFGF has been shown to increase cAMP accumulation in response to FSH or forskolin (Adashi *et al.* 1988a; LaPolt *et al.* 1990c), and to stimulate plasminogen activator expression, progesterone production (Baird & Hsueh, 1986), oocyte maturation and prostaglandin production (LaPolt *et al.* 1990c). Decreased androgen, oestrogen and inhibin production and increased progesterone, prostaglandin and plasminogen activator production, associated with oocyte maturation, are all events which take place during ovulation and luteinisation. Together with the established angiogenic effects of FGFs, and their lack of effect on granulosa cell proliferation in the rat, these results strongly implicate FGFs in ovulation and establishment of the corpus luteum in this species. However, the fact that granulosa cells of several species including humans, pigs, rabbits and guinea pigs grow in response to FGF (Gospodarowicz & Bialecki, 1979; Tapanainen *et al.* 1987) suggests that the rat is not a representative model for the study of the

regulation of granulosa cell proliferation. Furthermore, FGF has no effect on the differentiation of pig, cow and human granulosa cells (Tapanainen *et al.* 1987; Mondschein & Hammond, 1988; Urban *et al.* 1990; Schams *et al.* 1988), except at high doses in the pig (May *et al.* 1987; Biswas *et al.* 1988). FGFs, mainly bFGF, are probably most important for stimulating the vascularisation of the theca layer and the corpus luteum, and their direct effects on granulosa cells depend on the species.

6.2.8 Other Factors

Because of the accessibility of the *in vitro* granulosa cell model, an extremely wide variety of different factors have been tested for potential regulatory effects. Such factors include platelet-derived growth factor (PDGF) (Hammond & English, 1987; May *et al.* 1990), nerve growth factor (Lara *et al.* 1990), cytokines such as tumour necrosis factor (Adashi *et al.* 1990; Andreani *et al.* 1991; Veldhuis *et al.* 1991) and interleukins (Adashi, 1989; Adashi, 1990; Kasson & Gorospe, 1989), components of the renin-angiotensin system (Itskovitz & Sealey, 1987; Do *et al.* 1988; Kim *et al.* 1987), substance P (Dees *et al.* 1985), vasoactive intestinal peptide (Davoren & Hsueh, 1985; Liu *et al.* 1987b), relaxin (Too *et al.* 1984) and arginine vasopressin (Khan Dawood & Dawood, 1989; Verges *et al.* 1986). Many of these factors, and possibly several so far unknown factors as well, will inevitably affect ovarian function *in vivo*, but there is not enough information to speculate on the nature and extent of their involvement in the control of ovarian function.

7 Summary

At the beginning of the ovarian cycle, a large number of primary follicles begin to grow under the control of the gonadotrophins, FSH and LH. Of these follicles, only a small number (which depends upon the species) will ovulate at midcycle. Growth of small follicles is initiated by a rise in circulating levels of FSH which acts on the granulosa cells of the follicle, causing them to proliferate, and also to differentiate into active steroidogenic cells whose major product is oestradiol. A number of other

functional changes take place during granulosa cell differentiation, including the induction of receptors for LH, and the production of inhibin. As granulosa cells differentiate in response to FSH, their output of oestradiol, and also inhibin, increases. Oestrogen feeds back to the pituitary, where it suppresses FSH secretion, leading to a drop in circulating levels of FSH. Inhibin may also participate in this negative feedback loop. The majority of the growing follicles are unable to withstand this fall in trophic support, and degenerate. A number of these follicles, however, continue to grow in spite of the fall in FSH levels, and will ultimately ovulate. Since all follicles are exposed to the same concentrations of gonadotrophins at any one time, it is likely that there are mechanisms operating at the local level which determine whether a follicle is selected or becomes atretic. It is thought that the fate of a follicle depends upon the sensitivity of its granulosa cells to FSH and also LH, and that this is a function of the degree of differentiation of these granulosa cells. A variety of factors have been implicated in this local control of granulosa cell differentiation, in particular locally produced sex steroids, which have been shown to augment strongly the actions of FSH on some aspects of granulosa cell differentiation. This interaction between FSH and locally produced androgen and oestrogen in the control of granulosa cell differentiation was the subject of the studies described in this thesis.

8 Scope of the Thesis

Much is known about the effects of steroids on certain aspects of granulosa cell differentiation, in particular steroidogenesis and LH responsiveness, but few systematic studies have addressed their effects on gene expression and protein synthesis in these cells. In the course of this project, two main fields of study have been identified. Firstly, the role of steroids in the control of morphological differentiation at the level of cytoskeletal protein synthesis and gene expression was investigated. Secondly, the importance of ovarian protein hormones, in particular the inhibins and activins, as local as well as endocrine regulators of ovarian function is becoming increasingly recognised. Consequently, studies were

carried out to investigate the effects of steroids on the expression of the inhibin genes. In Chapter 2 the adaptation and validation of a rat granulosa cell culture system for the study of the effects of steroids on protein synthesis and gene expression is presented. Studies on steroidal regulation of cytoskeletal protein synthesis and gene expression are described in Chapter 3. The control of inhibin gene expression by steroids is presented in Chapter 4, and the gonadotrophic regulation of inhibin gene expression is correlated with changes in steroidogenic enzyme and LH receptor gene expression both *in vitro* and *in vivo*. Because little consensus has been reached on the regulation or even existence of steroid receptors in granulosa cells, studies on the expression of the androgen and oestrogen receptors were carried out, and the results of these studies are also presented in Chapter 2. Because of the limited material available from cultured cells, and the lack of quantitative information on RNA levels that can be gained from membrane hybridisation, the development of a sensitive and quantitative solution hybridisation assay for inhibin-α mRNA was undertaken, and is presented in Chapter 5.

Chapter 2 Progesterone Production and Protein Synthesis *In Vitro*

1 Introduction

Studies on the specific effects of hormones on a particular cell type require a convenient model system with clearly defined conditions. The model used throughout this thesis was a serum-free culture system using granulosa cells isolated from immature rats pretreated *in vivo* with diethylstilboestrol. This model has been in widespread use for a number of years, and much of what is known about the differentiation of granulosa cells has been gleaned using this model (Hsueh *et al.* 1984), which is based on the ability of oestrogen to stimulate proliferation of rat granulosa cells when administered *in vivo* (Simpson *et al.* 1941), to yield large numbers of granulosa cells from preantral and small antral follicles, and therefore in an undifferentiated state.

The purpose of this part of the project was to identify some of the changes in protein synthesis and gene expression which accompany granulosa cell differentiation in response to FSH and steroids. The steroids of particular interest were not only oestrogen, but also their precursors (androgens), and metabolites (catecholoestrogens). Since granulosa cell progesterone production has been widely used as a marker of differentiated function *in vitro* (Armstrong & Dorrington, 1976; Welsh *et al.* 1983; Hudson *et al.* 1987) and is easily measured by radioimmunoassay, this was the criterion by which the effects of FSH and steroids on granulosa cell differentiation, and their dose-response characteristics, were initially evaluated before studies on protein synthesis were undertaken.

Progesterone production was also used as a marker for hormonal responsiveness during the optimisation of an existing rat granulosa cell culture system, which is also described in this chapter. This system, in which cells are cultured for 48h at a plating density of 2.5×10^4 cells per

well, was developed for the study of the hormonal regulation of granulosa cell steroidogenesis and cAMP formation (Hillier & deZwart, 1982; Hudson *et al.* 1987). However, since other end-points were to be studied in this project, experiments were carried out to determine the optimum incubation time for the study of hormone-dependent changes in protein synthesis. The possibility of increasing the cell density was also explored, to improve yields of protein samples for analysis by electrophoresis and immunoprecipitation. Plating density has been shown to influence granulosa cell hormonal responsiveness *in vitro* (Davoren & Hsueh, 1986), so patterns of protein synthesis and progesterone production were observed over a range of cell densities.

The major aim of the project was to investigate the modulatory effects of oestrogen on FSH-induced granulosa cell differentiation. The use of oestrogen to stimulate proliferation of cells *in vivo* may affect their subsequent responses to oestrogen and other steroids *in vitro*, so experiments were also carried out to establish the effects, if any, of DES treatment *in vivo* on the effects of steroids *in vitro*. In addition, most culture media include phenol red as a pH indicator. This compound has been shown to exhibit weak oestrogenic activity in cultures of MCF-7 cells (Berthois *et al.* 1986; Welshons *et al.* 1988), and therefore has the potential to compromise the defined conditions of the culture system. Therefore, the effects of phenol red on granulosa cell steroidogenesis were investigated.

As discussed in chapter 1, section 6.1, there is still some doubt concerning the existence of steroid receptors in granulosa cells, and therefore the specificity of the effects of steroids. One way in which specificity can be demonstrated is by the use of competitive antagonists. A number of studies have shown clear reversal of the augmentation by androgens of FSH-stimulated differentiation using the antiandrogen hydroxyflutamide (Hillier & deZwart, 1981; Hillier & deZwart, 1982; Duleba *et al.* 1985), suggesting that these effects of androgens are mediated through the androgen receptor. However, the nonsteroidal antioestrogens tamoxifen and keoxifene have been shown to inhibit FSH- or cAMP-induced differentiation of cultured rat granulosa cells in the absence of oestrogen (Knecht *et al.* 1985; Kessel & Hsueh, 1987), suggesting

either that the antioestrogens were inhibiting the action of endogenous oestrogen produced in response to FSH or cAMP, or that the effects of the antioestrogens were nonspecific. Furthermore, these compounds are partial oestrogen agonists in this and other systems (Kessel & Hsueh, 1987; Wakeling *et al.* 1989), and have other effects as well as their antioestrogenic properties (Faye *et al.* 1987; Vonderhaar & Banerjee, 1991), making interpretation of results difficult. A "pure" antioestrogen (i.e. without agonistic activity), ICI 164,384, which is a 7α- tertiary amide derivative of 17β-oestradiol, has been used successfully in assays such as the inhibition of MCF-7 cell proliferation and oestrogen-stimulated rat uterine weight and progesterone receptor synthesis (Wakeling & Bowler, 1988; Wakeling *et al.* 1989). This compound was used to study the specificity of the effects of 17β-oestradiol on granulosa cell progesterone production, and the results of these experiments are described in this chapter.

An alternative approach to the investigation of steroid receptors in granulosa cells is to use nucleic acid probes to detect mRNA encoding these proteins. Despite demonstration of high affinity oestrogen binding sites in the nuclei of granulosa cells, studies using nucleic acid probes failed to detect oestrogen receptor mRNA in mouse ovaries, although a related RNA species was detected (reviewed in chapter 1, section 6.1.1). Similarly, expression of androgen receptor mRNA in granulosa cells has not been reported (reviewed in chapter 1, section 6.1.2). Therefore, a further aim of the studies described in this chapter was to investigate the expression of mRNA encoding the androgen and oestrogen receptors in rat granulosa cells, and to study the effects of gonadotrophins on the expression of these genes.

2 Materials and Methods

2.1 Hormones and Antagonists

Where FSH was administered to animals *in vivo*, 20μg of ovine FSH (NIADDK-o-FSH-16; FSH bioactivity = 20U/mg, LH bioactivity = 0.04 x NIH-LH-S1; donated by the National Institute of Arthritis, Metabolism

and Digestive Diseases, Bethesda, U.S.A.), in 100µl DPBS containing 0.1% BSA, was injected subcutaneously every 12 hours for the 48 hours prior to removal of the ovaries. Where hCG was administered *in vivo*, to mimic a surge of LH, 20IU hCG (purchased from Sigma Chemical Co., Poole, Dorset) were injected subcutaneously in 100µl DPBS containing 0.1% BSA, 12h before animals were killed.

Human (h) FSH, which was the generous gift of Prof. L.E. Reichert, Jr. (LER 8/116; FSH bioactivity = 900U/mg, LH bioactivity = ~0.5IU/mg) was stored at -20C in 0.1% BSA in DPBS, and was used for all *in vitro* experiments at final concentrations of 3-200ng/ml. hCG was also stored at -20C in DPBS containing 0.1% BSA, and was added to cultures at a final concentration of 200mIU/ml. Stock solutions (10^{-6}M to 10^{-3}M) of Testosterone (T; 17β-hydroxyandrost-4-en-3-one), 17β-oestradiol (E_2; 1,3,5,[10]-oestratrien-3,17β-diol), 5α-dihydrotestosterone (DHT; 17β-hydroxy-5α-androstan-3-one) and 2-hydroxyoestradiol (2-OH-E_2; 1,3,5[10]-oestratriene-2,3,17β-triol), all purchased from Sigma, were stored at -20C in ethanol. Steroids were added to culture wells in a volume of 5µl, to give final concentrations of 10^{-9}M to 10^{-5}M. The antioestrogen ICI 164,384 (N-*n*- butyl - 11 - [1,3,5(10)-oestratriene-3,17β-diol -7α- yl] -N-methylundecanamide) and the inactive 7β-isomer of the secondary amide, ICI 164,275 were donated Dr. A. Wakeling, ICI Pharmaceuticals, Macclesfield, Cheshire (see Fig.2.1 for structures). Stock solutions of these compounds were stored in ethanol at -20C. Phenol red was purchased from Sigma.

2.2 Animals

Immature female Wistar and Sprague-Dawley rats were used for the experiments presented throughout this thesis. These animals were either bred in-house or purchased from Bantin and Kingman Ltd., Hull. The animals were 21 days old at the beginning of the experiments. Animals were treated with DES (Sigma) using 1cm silastic tubing capsules containing solid DES, which were implanted subcutaneously under general anaesthesia in the neck region of the animals. These capsules were left in place for 4 days. Adult mice (hybrids of CBA and C57BL

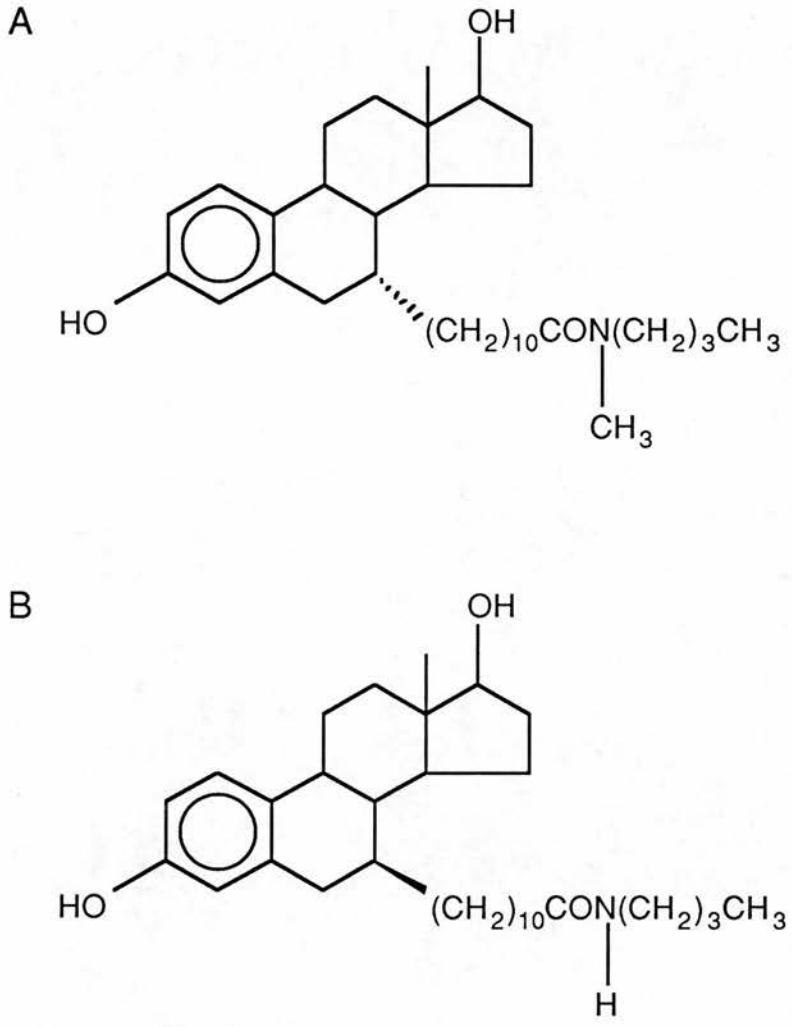


Fig.2.1 A. Structure of the antioestrogen ICI 164,384 (N-*n*-butyl-11-(1,3,5[10]-oestratriene-3,17β-diol-7α-yl)-N-methylundecanamide). B. Structure of the 7β-isomer of the secondary amide derivative, ICI 164,275 (N-*n*-butyl-11-(1,3,5[10]-oestratriene-3,17β-diol-7β-yl) undecanamide).

strains, 40 days old) were also used for studies of oestrogen receptor mRNA expression.

2.3 Cell Culture Method

Animals were killed with carbon dioxide or by cervical dislocation. Ovaries were removed and dissected free from fat and other surrounding tissue. Granulosa cells from preantral and early antral follicles were harvested in culture medium by gentle puncture with a hypodermic needle. Cells were then centrifuged at 1500rpm for 10min and resuspended in 1ml of medium. To disperse the cells and to eliminate clumps, the suspension was drawn through a pipette tip several times, made up to 10ml with medium, and allowed to settle for 1min. The cells remaining in suspension were then centrifuged, resuspended and triturated again as before. An aliquot of the cell suspension was removed and mixed with an equal volume of trypan blue solution, and cells in this mixture were counted using a haemocytometer. Viability was assessed based on the exclusion of the dye, and was typically >40%. Yields of granulosa cells were 2.03 (±0.11) $x10^6$ cells per DES-treated animal (29 experiments), or 0.95 (±0.07) $x10^6$ cells per control animal (13 experiments). Cells were cultured in Medium 199 containing 25 mM HEPES buffer, extra (2 mM) L-glutamine, penicillin (50 IU/ml) and streptomycin (50 mg/ml) (all from Gibco Ltd, Paisley, UK) with 0.1% (w/v) BSA (ICN Biomedicals, High Wycombe, Bucks, UK). Multiwell plastic dishes (Linbro Space Savers, Flow Laboratories, Rickmansworth, Herts, UK) were precoated with donor calf serum (Gibco) and washed twice with 1 ml Dulbecco's phosphate buffered saline (DPBS; Oxoid) before use. The culture wells were inoculated with replicate 250 µl portions of cell suspension containing the required number of viable granulosa cells. The cultures were incubated at 37C in a humidified incubator gassed with 95% air/5% CO_2. Cultures were incubated for 48h for most experiments. To study the time-course of hormonal responses, however, incubations of 6, 24, 48 and 72h duration were also carried out. Metabolic labelling incubations (see below) lasted for 24h in all cases.

To investigate the effect of cell plating density on hormonal responsiveness and protein synthesis, granulosa cells were cultured at densities of 50, 100, 150, 200, 300 and 400x10^3 cells per well. After 48h in culture, medium was removed and the monolayers were washed once with 1ml of Minimum Essential Medium (MEM; Gibco), and cells were recovered from some of the culture wells by incubation at room temperature for 10min in 250µl trypsin/EDTA solution (0.5mg/ml trypsin and 0.5mM EDTA in Modified Puck's Saline A; Gibco). 250 µl of MEM containing 5% donor calf serum was added to each well, to prevent over-digestion with trypsin, and cells were transferred to microcentrifuge tubes. Wells were washed out with 500µl MEM, which was pooled with the cells. Cells were pelleted at 1500 x g for 10min, and gently resuspended in 250µl DPBS. Cells were then counted in a haemocytometer.

2.4 Progesterone Radioimmunoassay

After the culture period, media were removed and stored at -20C until required. Progesterone (4-pregnene-3,20-dione) was measured in culture media by specific radioimmunoassay. The assay buffer was phosphate buffered saline/gelatine (0.05M sodium phosphate, 0.15M NaCl, 0.1% gelatine). The antibody, raised in the rabbit against progesterone-11α-hemisuccinate-BSA (R31/12), was provided by MRC, Mill Hill. Antibody was used at a working dilution of 1:4,500. Cross-reaction of the antibody with oestradiol-17β, ICI 164,384, ICI 164,275 or SCH 16423 was <0.01%. Cross-reaction with testosterone, 5α-dihydrotestosterone and 2-hydroxyoestradiol was 0.04%, 0.05% and 0.02%, respectively. Tracer, [1,2,6,7-^{3}H]-Progesterone (80-110Ci/mmol, Amersham), was stored at -20C in ethanol, at a concentration of 10µCi/ml. Before use this stock was diluted 1:400 in assay buffer to give ~10,000cpm/500µl. A standard curve covering the range of 12.5 to 1600pg/tube 4-pregnene-3,20-dione (Sigma), made up in culture medium, was used. Samples (5-100µl) or standards (in 200µl) were placed in tubes, and the volume of samples was adjusted to 200µl. Quality controls (83 and 850pg/tube) were also included at the beginning and end of each assay. Antibody (300µl) and tracer (500µl) were added, and tubes were

incubated overnight at 4C. Tubes were placed on ice for 15min before addition of 200µl dextran charcoal (1.25% dextran, 1.25% charcoal in assay buffer) to separate bound and free progesterone. Tubes were mixed and incubated for 10min on ice, then centrifuged at 3000rpm for 10min at 4C. Supernatants were decanted, 3ml scintillation cocktail (RIALUMA, Lumac LSC b.v., Olen, Belgium) was added, and bound tracer was measured by liquid scintillation counting (RackBeta, LKB Wallac, Turku, Finland). Standard curves were calculated using a commercial computer program (AssayZap, Biosoft, Cambridge, UK). Samples which were outwith the detection limits were re-assayed at the appropriate volume. Specific binding (B/B_0) was 38-43%, and nonspecific binding was 2-4%. The detection limit of the assay was in the range 15-20pg/tube. Inter- and intra-assay coefficients of variation were 15% and 6%, respectively.

Statistical analysis was carried out using commercial software (CLR ANOVA, Clear Lake Research Inc., Houston, TX, USA). Analysis of variance was carried out on results after log transformation to reduce heterogeneity of variation, and statistical differences at the $p<0.05$ level were revealed using Duncan's Multiple Range Test.

2.5 Analysis of Protein Synthesis

2.5.1 Metabolic Labelling

In order to study the effects of *in vitro* treatments on patterns of protein synthesis, cultures were incubated as above in the presence of test agents, and monolayers were washed once with 1ml MEM before being incubated for an additional 24h in 250µl methionine-free MEM containing extra (2 mM) L-glutamine, penicillin (50 IU/ml) and streptomycin (50 mg/ml), but without BSA. 10-20 µCi ^{35}S-methionine (Tran^{35}S-Label, ICN) was then added to each culture well. The test agents treatments were also included in the medium during this labelling period. In an experiment to study the effects of FSH administration *in vivo* on the patterns of granulosa cell protein synthesis, cells were isolated as described above, but were resuspended in methionine-free MEM containing ^{35}S-methionine, and cultured for 24h.

The media from metabolically labelled cultures were removed after the labelling period, replicates being pooled, and aprotinin (Sigma) was added to a final concentration of 15 KIU/ml. The cell monolayers from those cultures were lysed with a solution containing 20mM Tris-HCl (pH6.8), 0.5% (v/v) NP-40, 40 KIU/ml aprotinin, 2 mM phenyl-methyl-sulphonylfluoride (PMSF) and 0.02% (w/v) sodium azide (all from Sigma). Cells were scraped off the bottom of the wells with a pipette tip and replicates were pooled. Media and cell extracts were centrifuged at 13,000g for 5min to remove cell debris, and supernatants were transferred to fresh tubes, and stored at -20°C until required.

2.5.2 TCA Precipitation

The amount of radioactive label incorporated into protein in the samples was determined by precipitation of the proteins with trichloroacetic acid (TCA) according to the method of Kessler (1981). Triplicate aliquots of each sample were mixed with 30µl of a solution of 10mM sodium phosphate pH6.5 and 130mM sodium chloride containing 10mg/ml BSA as a carrier protein. 1ml of 10% (w/v) TCA containing 10mM L-methionine was added to each tube and tubes were placed on ice for 30min. Samples were centrifuged at 13000g for 5 min and the supernatants were aspirated. Pellets were taken up in 200µl 0.1M potassium hydroxide, and a further 1ml of TCA was added. Tubes were placed on ice, centrifuged and supernatants aspirated again as before. Final pellets were taken up in 100µl 0.5M KOH, and 100µl of each sample was counted by liquid scintillation counting.

2.5.3 One Dimensional SDS-PAGE

Radiolabelled proteins were routinely separated according to their molecular weight by electrophoresis in polyacrylamide gels containing sodium dodecyl sulphate (SDS), according to the method of Laemmli *et al.* (1970). A BioRad Mini Protean II minigel system was used for this purpose.

Either 12.5% single percentage or 7.5%-15% polyacrylamide gradient slab gels were used, containing 375mM Tris (pH8.8) and 0.1% SDS (w/v). The ratio of acrylamide:bisacrylamide used was 37.5:1, and gels were prepared from a stock solution of 30% acrylamide (w/v) and 0.8% bisacrylamide (w/v). Gel mixtures were polymerised with 0.05% (v/v) N,N,N',N'-tetramethylethylenediamine (TEMED, Sigma) and 0.05% (w/v) ammonium persulphate (APS, BDH). Gradient gels were prepared using a gradient former, and the mixture was pumped into a BioRad Mini Protean II multi-casting chamber, forming up to 11 gels at one time which could be stored until needed. Gels were overlaid with water-saturated butanol until polymerisation was complete. In all cases discontinuous gels with a stacking gel were used. The composition of the stacking gel was 4% acrylamide, 125mM Tris-HCl (pH6.8) and 0.1% (w/v) SDS.

Samples were prepared by adding an equal volume of denaturing sample buffer (40mM Tris-HCl [pH6.8], 2% [w/v] SDS, 10% [v/v] glycerol and 5% [v/v] β-mercaptoethanol [βME]) to an aliquot of each sample and boiling for 5min. Samples were then centrifuged at 13,000xg for 5min, and supernatants transferred to fresh tubes.

Equal counts of radioactive protein samples as determined by TCA precipitation (10-20x10^3 cpm) were loaded onto polyacrylamide gels in a volume of 0.5-50µl. Molecular weight standards (Sigma) were also loaded, and electrophoresis was carried out in an electrode buffer containing 25mM Tris (pH8.3), 200mM glycine and 0.1% (w/v) SDS at 180V until the dye-front had reached the end of the gel (approximately 45min). Proteins were then stained by soaking gels for 1h in 1.5% (w/v) Coomassie brilliant blue (Sigma) in 20% (v/v) ethanol and 8% (v/v) acetic acid and gels were destained overnight in acid ethanol alone to visualise molecular weight markers. Radiolabelled proteins were visualised by autoradiography or fluorography of dried gels. Gels containing 20x10^3 cpm or more of radioactive proteins were exposed to Amersham β-Max film at room temperature (direct autoradiography), whereas gels containing less radioactivity were soaked in Amplify (Amersham) for 5min before drying, and then exposed to Kodak XAR-5 film at -70C (fluorography).

2.5.4 Two Dimensional Electrophoresis

To reveal the effect of FSH on patterns of proteins secreted by granulosa cells *in vivo*, proteins secreted by cells freshly isolated from control and FSH treated animals were analysed by two dimensional polyacrylamide electrophoresis, according to the method of Hochstrasser *et al.* (1988), using a Bio-Rad Mini-Protean II 2-D cell. First dimension (isoelectric focussing) tube gels were prepared, containing 4% acrylamide, 9M urea, 2% (v/v) NP-40 and 2% (v/v) ampholines (from a 7:1 mixture of Biolytes pH 7-9 and 3-10, Bio-Rad). The mixture was thoroughly degassed before being polymerised with 0.02% APS and 0.1% TEMED and overlaid with distilled water. After polymerisation had been allowed to take place, the overlay was replaced with degassed 20mM NaOH. After a further 30min, this was replaced with fresh 20mM NaOH. Gels were prefocussed at 200V for 10min, 300V for 15min, and 400V for 15min. The upper chamber buffer was 20mM NaOH, and the lower chamber buffer was 6mM H_3PO_4. Samples were mixed with an equal volume of solubilising/reducing buffer (9M urea, 2% NP-40, 2% ampholines and 1% [w/v] dithiothreitol [DTT]), and additional urea was added to a final concentration of 9M. Samples were incubated at room temperature for 15min and centrifuged at 13,000 x g for 3min. Supernatants were transferred to fresh tubes, and $1x10^5$ cpm of total radiolabelled proteins were loaded onto the first dimension gels immediately. Blank gels, loaded with diluted sample buffer only, and gels loaded with isoelectric point standards (IEF Mix 3.6-9.3, Sigma) were also run, to calibrate the pH separation range. Isoelectric focussing was carried out at 500V for 2.5h, 750V for 1h, and 990V for 30min. Samples were separated in the second dimension according to molecular weight as described in the previous section, after equilibration of first dimension gels for 10min in denaturing sample buffer. Proteins were detected by fluorography as described above.

2.6 Analysis of mRNA

2.6.1 Extraction, Purification and Quantitation of Total Cellular RNA

Total RNA was extracted from granulosa cells according to the method of Chomczynsky and Sacchi (Chomczynski & Sacchi, 1987). Medium was removed from granulosa cell cultures, and the cells were lysed on ice with cold "solution D" (4M guanidinium thiocyanate [Fluka, Glossop, Derbyshire], 25mM sodium citrate, 0.5% [w/v] sarcosyl, 100mM β-mercaptoethanol; 200µl/well). The cell lysate was transferred to polypropylene tubes (Falcon 2059, Becton Dickinson, Cowley, Oxford), and an additional 100µl of solution D was added to each well, and pooled with the initial lysate. One tenth of a volume of 2M sodium acetate (pH4), one volume of water-saturated phenol and one fifth of a volume of chloroform:isoamyl alcohol (49:1) were added, tubes being capped and shaken between additions. Tubes were shaken vigorously for 10sec after the last addition, and placed on ice for 15min. Tubes were then centrifuged at 10,000g for 20min at 4C to separate the phases. The aqueous (top) phases were transferred to fresh tubes, taking great care to leave behind the protein present at the interface, and an equal volume of cold isopropanol was added. Tubes were left at -20C for at least 1h to allow the RNA to precipitate. Precipitated RNA was recovered by centrifugation at 10,000g for 20min at 4C. The supernatants were poured off, pellets were dissolved in 300µl of solution D, and transferred to microcentrifuge tubes. RNA was reprecipitated with an equal volume of isopropanol at -20C for at least 1h, and then recovered by centrifugation at 13,000g for 10min at 4C. RNA pellets were washed with 70% (v/v) ethanol and allowed to dry by leaving tubes open at room temperature, before being dissolved in 30µl of 0.5% (w/v) SDS.

Concentration and purity of RNA preparations were determined by spectrophotometry. A small aliquot of each preparation was diluted with water, and transferred to a spectrophotometer cuvette. The purity of an RNA sample was calculated from the ratio of the absorbance of the solution at 260nm (A_{260}) to its absorbance at 280nm (A_{280}). A ratio of 2.0

was considered optimal purity. Any samples with an A_{260}:A_{280} ratio of less than 1.5 were re-extracted with an equal volume of phenol:chloroform and reprecipitated with 0.1 volume of 3M sodium acetate and 2.5 volumes of ethanol at -20C for at least 1h, then recovered by centrifugation, taken up in 0.5% SDS as before, and scanned in the spectrophotometer once again. Concentration of RNA samples was calculated from the A_{260}. A solution containing 40mg of RNA/ml has an A_{260} of 1.0. A_{260} values of samples were therefore multiplied by 40mg/ml, and corrected for the dilution factor to give the concentration of RNA in the original sample. One tenth of a volume of 3M sodium acetate and 2.5 volumes of ethanol were added to the samples, and they were stored at -70C until required.

2.6.2 *Electrophoresis and Northern Blotting*

Equal amounts of total RNA from each sample were centrifuged at 13,000g for 10min, washed with 1ml 75% (v/v) ethanol, and pellets were allowed to dry at room temperature. RNA was redissolved in 4.4µl 0.5% (w/v) SDS by heating to 65C for 10min. Buffer (15.6µl) was added to a final concentration of 20mM 3-(N-morpholino) propanesulphonic acid (MOPS), 5mM sodium acetate, 1mM EDTA, 50% formamide, 18% formaldehyde, and 50µg/ml ethidium bromide, and samples were heated to 60C for a further 5min, to denature RNA, and placed immediately on ice. Two microlitres of dye (25% [w/v] Ficoll 400 [Pharmacia], 0.25% bromophenol blue, 1mM EDTA) was added, samples were vortexed briefly, and centrifuged for 5s, to bring the samples to the bottom of the tubes. Samples were immediately loaded onto gels containing 1.5% (w/v) agarose, 6.6% (v/v) formaldehyde in running buffer containing 20mM MOPS, 5mM sodium acetate, 1mM EDTA (pH7.0), and subjected to electrophoresis using a commercial horizontal gel electrophoresis tank (Bio Rad), in running buffer. After electrophoresis, gels were photographed under ultraviolet transillumination to check integrity and even loading of RNA, and to measure migration of RNA molecular weight standards and ribosomal RNA. RNA was partially hydrolysed by soaking gels in 0.05M NaOH for 20min, to improve RNA transfer. Gels

were then soaked for 45min in 20xSSC (3M NaCl, 300mM sodium citrate [pH7.0]), before RNA was transferred overnight to uncharged nylon membranes (Hybond-N, Amersham) by capillary blotting. RNA was fixed to Northern blots by exposure to ultraviolet radiation (260nm) for 5min.

2.6.3 Preparation of Plasmids

Plasmid containing cDNA encoding the mouse oestrogen receptor (White *et al.* 1987) was obtained from Dr. M.G.Parker, ICRF, Lincoln's Inn Fields, London. The 1.9kb insert was cloned into the *Eco*RI site of the plasmid pSP64. The orientation of the insert was such that SP6 RNA polymerase could be used to transcribe antisense RNA probes.

The rat androgen receptor cDNA (Chang *et al.* 1988) was obtained from Dr.S.Liao, Chicago, Illinois, USA. The 2830bp insert was cloned between the *Eco*RI and PstI sites of the plasmid pGEM3Z.

Plasmids were amplified by transfection of JM109 strain *E. coli* (Promega), which were rendered competent for transfection by the following procedure. Cells were streaked on M-9 plates containing thiamine-HCl (42mM Na_2HPO_4, 22mM KH_2PO_4, 20mM NH_4Cl, 9mM NaCl, 2mM $MgSO_4$, 1mM thiamine-HCl, 100µM $CaCl_2$, 1.5% [w/v] agarose, 0.2% [w/v] glucose) and incubated overnight at 37C. A single colony was picked, and used to inoculate 25ml of Luria-Bertani medium (LB; 1% [w/v] bactotryptone, 0.5% [w/v] bacto-yeast extract [both from Difco,], 0.5% [w/v] NaCl) which was then shaken at 225rpm overnight at 30C. Five hundred millilitres of LB medium were inoculated with 5ml of this culture and shaken at 150rpm until the absorbance of the culture at 600nm reached 0.5. The culture was placed on ice water for 2h, and the cells collected by centrifugation at 2500g for 15min at 4C. The cells were gently resuspended in 20ml of ice-cold trituration buffer (100mM $CaCl_2$, 70mM $MgCl_2$, 40mM sodium acetate [pH5.5]) and the volume was made up to 500ml with the same solution. The cells were then incubated on ice for 45min before being centrifuged at 1800g for 10min and gently resuspended in 50ml of ice-cold trituration buffer. 80% (v/v) glycerol was added to the suspension, dropwise with gentle swirling, to a final

concentration of 15%. The cells were divided into 1ml aliquots, snap-frozen in an ethanol-dry ice bath and stored at -70C.

Purified plasmids were introduced into the cells by a heat-shock transfection method. Aliquots of competent cells were thawed on ice and 100µl of cell suspension were pipetted on ice into pre-chilled Falcon 2059 15ml polypropylene tubes. Purified plasmids (10ng) were added to the cells on ice, moving the pipette tip through the cell suspension to mix, and tubes left on ice for 30min. Tubes were heated to 42C for 45sec, and then placed on ice again for 2min. 900µl of room temperature SOC medium (2% [w/v] bactotryptone, 0.5% [w/v] bacto-yeast extract, 10mM NaCl, 10mM $MgSO_4$, 10mM $MgCl_2$, 2.5mM KCl, 20mM glucose) was added, and tubes placed at 37C and shaken at 225rpm for 1h. 100µl of transformed cells were spread on LB agar (as LB medium, containing 1.5% [w/v] agar [Difco]) plates, containing 100µg/ml ampicillin, 40µg/ml X-Gal and 500µM IPTG. Plates were incubated overnight at 37C. White recombinant colonies were picked using a flamed inoculating loop and used to inoculate 5ml aliquots of LB medium containing 50µg/ml ampicillin in sterile Falcon 2059 tubes. The tubes were incubated for at least 6h at 37C with shaking at 225rpm. Cells were recovered from 1.5ml of these cultures by centrifugation at 13,000g for 30sec, and the supernatants discarded. Plasmids were recovered from the bacteria by resuspension in 110µl STETL (50mM Tris [pH8.0], 50mM EDTA, 8% [w/v] sucrose, 5% [v/v] Triton X-100, 0.5 mg/ml lysozyme), and incubation at room temperature for 10min. The suspensions were boiled for 2min, and then centrifuged at 13,000g for 15min. The gelatinous pellets were then removed with sterile pipette tips, and 110µl isopropanol was added to the supernatants at room temperature, and the samples were immediately centrifuged at 13,000g for 15min. The pellets of DNA were then washed with 70% ethanol, and allowed to dry. After dissolving the pellets in 30µl TE buffer (10mM Tris [pH7.5], 1mM EDTA), the plasmids were stored at -20C. In order to confirm that clones contained the required plasmid, plasmid preparations were incubated with the appropriate restriction enzymes to liberate inserts, and plasmids and restriction digests were subjected to electrophoresis in 0.8% [w/v] agarose gels containing 1µg/ml ethidium bromide, using TAE (40mM Tris-acetate, 1mM EDTA) as the

electrode buffer, and visualised under ultraviolet light. Positive clones were then grown in large-scale culture. Cells transformed with plasmids of interest were used to inoculate 25ml cultures of LB medium containing 50μg/ml ampicillin, which were incubated overnight at 37C with shaking at 200rpm. Overnight cultures were used to inoculate 400ml cultures of LB medium containing 50μg/ml ampicillin, which were incubated at 37C with shaking at 150rpm until the absorbance at 600nm reached 0.8. Cloramphenicol was added to a final concentration of 170μg/ml, and cultures were incubated overnight at 37C with shaking at 150rpm. Bacteria were pelleted at 3000g for 10min, and plasmids were purified by an alkaline lysis method, using a commercial kit (Qiagen Maxi Prep kit, Hybaid). Bacterial pellets were resuspended in 10ml of a buffer containing 50mM **Tris**, 10mM EDTA, 100μg/ml RNase A. Ten ml of 200mM NaOH containing 1% [w/v] SDS were added, and the suspensions incubated at room temperature for 5min. Ten ml of 2.55M potassium acetate (pH4.8) were added, suspensions were immediately gently mixed and centrifuged at 20,000g for 30min at 4C. Supernatants were removed promptly, and centrifuged again at 20,000g for 10min at 4C, to obtain a clear lysate. Plasmids were purified from lysates by ion exchange chromatography. Columns (Qiagen tip 500) were equilibrated with 10ml of a buffer containing 50mM MOPS (pH7.0), 750mM NaCl, 0.15% (v/v) Triton X-100, 15% (v/v) ethanol, and the lysates applied by gravity. Columns were washed with 3x10ml of a buffer containing 50mM MOPS (pH7.0), 1M NaCl and 15% (v/v) ethanol, to remove RNA, protein and other impurities. Plasmids were eluted from the resin in 15ml of a buffer containing 50mM MOPS (pH8.2), 1.25M NaCl and 15% (v/v) ethanol, and recovered by precipitation with 0.7 volumes of isopropanol and centrifugation at 10,000g for 30min at room temperature. Pellets of plasmid DNA were washed with 10ml 70% (v/v) ethanol, allowed to dry, and redissolved in TE buffer. Concentration of plasmids was assessed by spectrophotometry as described for RNA above, except that for the calculation of concentration, an A_{260} of 1.0 was assumed to indicate a concentration of 50μg/ml double-stranded DNA. Plasmids were stored at -20C in TE buffer, at a concentration of 5mg/ml.

2.6.4 Preparation of ^{32}P-labelled cDNA Probes

cDNA inserts were liberated from purified plasmids by incubation with restriction enzymes. The rat androgen receptor cDNA contains an internal *Eco*RI site, so that restriction of the plasmid with *Eco*RI and *Pst*I liberated the full cDNA in two fragments of approximately 2.4kb and 0.4kb. The reaction mixture was subjected to electrophoresis in a 0.8% agarose TAE gel as described above, and the two insert fragments were excised from the gel. A commercial kit (Geneclean II, Bio 101, Stratech Scientific, Luton, Beds) was used to purify DNA from gel slices. Gel was melted by incubation in three volumes of a solution containing 6M sodium iodide at 50C for 5min. 10µl of a suspension of silica matrix (Glassmilk) to which DNA binds in high salt concentrations was added, and tubes were incubated at room temperature for 5min with mixing. The silica-DNA complex was then centrifuged for 5sec in a microcentrifuge, and washed three times with 400µl of a buffer (NEW Wash, stored at -20C), containing NaCl, Tris, EDTA and 50% (v/v) ethanol. DNA was recovered by elution into TE buffer at 50C for 3min. The eluate was extracted once with phenol:chloroform (1:1), and precipitated with 0.1 volume of 3M sodium acetate (pH7.0) and 2.5 volumes of ethanol at -20C for at least 1h. Concentration of recovered cDNA was assessed by electrophoresis of aliquots of eluates in 0.8% agarose gels containing ethidium bromide as described above, and comparison of staining with that of standards of known concentration (1kb DNA ladder, BRL). Purified inserts were stored at -20C in TE buffer at a concentration of 5-10µg/ml.

cDNA probes were labelled by random priming using a commercial kit (Multiprime Kit, Amersham), based on the method of Feinberg and Vogelstein (1983). Approximately 25-50ng of purified cDNA was denatured by boiling for 2min, and placed on ice. Water was added to give a final reaction volume of 50µl, and 10µl of labelling buffer (unlabelled dGTP, dATP and dTTP in a buffer containing Tris [pH7.8], $MgCl_2$ and β-mercaptoethanol), 5µl random sequence hexamer primers (in a buffer containing BSA) and 50µCi of labelled dCTP (deoxy-cytidine 5'-[α-^{32}P] triphosphate; specific activity ~3000 Ci/mmol,

Amersham) were added on ice in order. Klenow DNA polymerase (2U) was added, the components were mixed by gentle passage through a pipette tip, and centrifuged briefly to the bottom of the tube, and the reaction mixture was incubated at 37C for 30min. Incorporation of labelled nucleotide and specific activity of probe were measured by adsorption of DNA to charged paper filters (DE81, Whatman). Duplicate 1µl aliquots of the reaction mixture were spotted onto DE81 filters and allowed to dry. One filter was washed five times for 5min in ~200ml 0.5M Na_2HPO_4 (pH7.0), then rinsed in 70% ethanol and allowed to dry. Both filters were placed in scintillation vials with 4ml of scintillation fluid, and radioactivity bound to the filters was measured by liquid scintillation counting. Counts bound to the washed filter represented dCTP incorporated into DNA, and those on the unwashed filter represented the total input of dCTP. Incorporation and specific activity were calculated to be >80% and ~10^9cpm/µg respectively (assuming approximately equal proportions of all four nucleotides in the template sequence). Labelled cDNA was separated from unincorporated nucleotides by chromatography on Sephadex G-50 columns (Nick Columns, Pharmacia) in TE buffer, and yield of probe was measured by scintillation counting. Denatured, fragmented herring sperm DNA (1mg; Promega) was added to the labelled probe fraction, and 100µl 5M NaOH was added, to denature DNA. Denatured probe was neutralised by the addition of 600µl 1M Tris (pH7.4) and 475µl 1N HCl (dropwise). Hybridisation buffer was added to give ~10^6cpm/ml antisense DNA probe.

2.6.5 *Preparation of ^{32}P-labelled cRNA Probes*

Oestrogen receptor mRNA was analysed by Northern hybridisation with a complementary RNA probe, transcribed *in vitro* from the mouse oestrogen receptor cDNA. Template was prepared by digestion of plasmid with *Bgl*II. In order to ensure that plasmid was fully linearised, restriction digestion was carried out overnight at 37C. An aliquot of digested DNA was subjected to agarose gel electrophoresis and visualised under UV light. A single band of DNA was observed, indicating that plasmid was fully linearised, and its concentration was estimated by comparison with

standards of known concentration. Template DNA was precipitated with 0.1 volume of 3M sodium acetate (pH7.0) and 2 volumes of ethanol at -20C for at least 1h, and centrifuged at 13,000g for 10min. The DNA pellet was redissolved in TE buffer to give a concentration of ~1mg/ml. Transcription of RNA from this template using SP6 RNA polymerase results in synthesis of RNA probes complementary to the portion of mRNA encoding the steroid-binding domain of the oestrogen receptor (Hillier *et al.* 1989a). *In vitro* transcription was carried out using a commercial kit (Riboprobe Gemini System II, Promega). One microgram of template DNA was added to a tube containing 40mM tris (pH7.5), 10mM NaCl, 6mM $MgCl_2$, 2mM spermidine, 10mM DTT, 20U ribonuclease inhibitor (RNasin, Promega), unlabelled ATP, UTP and CTP at 500μM each, and 12μM unlabelled GTP. Fifty microcuries of ^{32}P-labelled GTP (specific activity ~400Ci/mmol; final concentration, 6μM, Amersham), and 20U SP6 RNA polymerase were added, the components were mixed gently by passage through a pipette tip and brought to the bottom of the tube by brief centrifugation, and the reaction was allowed to take place for 1h at 37C. Incorporation and specific activity were calculated as described in section 2.6.4, and were >85% and ~2x10^8cpm/μg, respectively. Template DNA was removed by incubation with 2U RNase-free DNase (RQ1 DNase, Promega) for 15min at 37C. RNA probes were separated from unincorporated NTPs and degraded template by size exclusion chromatography through Sephadex G-50 columns (Nick Column, Pharmacia) in TE buffer containing 0.1% (w/v) SDS. Probe (10^6cpm/ml) was added to hybridisation buffer containing 100μg/ml denatured, fragmented herring sperm DNA.

2.6.6 Northern Hybridisation with ^{32}P-labelled Probes

Northern blots were prehybridised by incubation for at least 1h in hybridisation buffer (7% [w/v] SDS, 1% [w/v] BSA, 200mM sodium phosphate [pH7.2], 1mM EDTA), containing 15% [v/v] formamide, for hybridisation with cDNA probes, or 50% [v/v] formamide, for hybridisation with cRNA probes, using a commercial hybridisation oven (Hybaid). Prehybridisation buffer was replaced with probe solution.

Prehybridisation and hybridisation were carried out at 55C in buffer containing 15% formamide, and at 42C in buffer containing 50% formamide. Blots were washed twice for 30min at 65C in a large volume of washing buffer (40mM sodium phosphate [pH7.2], 1mM EDTA, 1% [w/v] SDS), then wrapped in cling film (Saran Wrap, GRI, Dunmow, Essex), and exposed to X-ray film (Kodak X-Omat AR-5, Sigma), using two intensifying screens (GRI).

3 Results

3.1 Responses to FSH and Steroids

3.1.1 Progesterone Production

Figure 2.2A shows progesterone secretion of cells from immature animals pretreated *in vivo* only with DES, and cultured in the presence or absence of 100ng/ml FSH or 0.2 IU/ml hCG. Basal progesterone secretion by these cells was low, and was moderately (approximately 4-fold) stimulated by treatment *in vitro* with FSH. Although all cells were isolated from small follicles, hCG also caused a small but significant increase in progesterone production.

Figure 2.2B shows the results of a similar experiment, using cells from animals pretreated *in vivo* with DES and FSH. Basal progesterone production by these cells was much greater than basal or stimulated progesterone production by control cells (see Fig.2.2A; note different scale). Treatment of these cells *in vitro* with FSH caused a slight stimulation of progesterone production, but treatment with hCG caused a very much greater stimulation, demonstrating the effect of FSH treatment *in vivo* in inducing LH/CG responsiveness.

The effect of FSH treatment of granulosa cells *in vitro* in stimulating progesterone secretion was further investigated by incubating cells in the presence of increasing doses of FSH. Fig 2.3 shows progesterone production by granulosa cells from immature animals pretreated with DES only, in response to 3-200ng/ml FSH. Note that at low to moderate concentrations (3-30ng/ml), FSH had little effect on progesterone

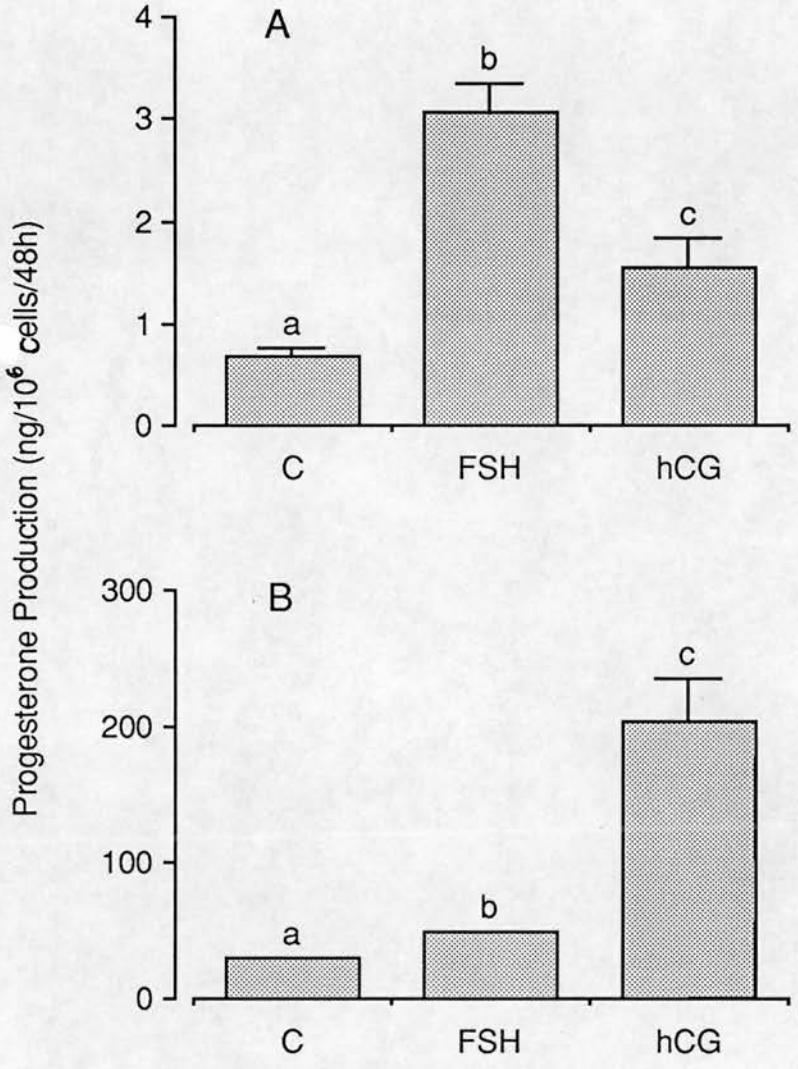


Fig.2.2 Progesterone production by rat granulosa cells cultured in the absence (C) or presence of 100ng/ml hFSH (FSH) or 200mIU/ml hCG (hCG). A. Animals were treated *in vivo* only with DES for 4 days before isolation of granulosa cells. B. Animals were injected with 20μg oFSH every 12 hours for 2 days, in addition to DES treatment before isolation of cells. Values represent the mean (±SE) from triplicate wells. In each graph, histograms with different superscripts are significantly different from one another ($p<0.05$).

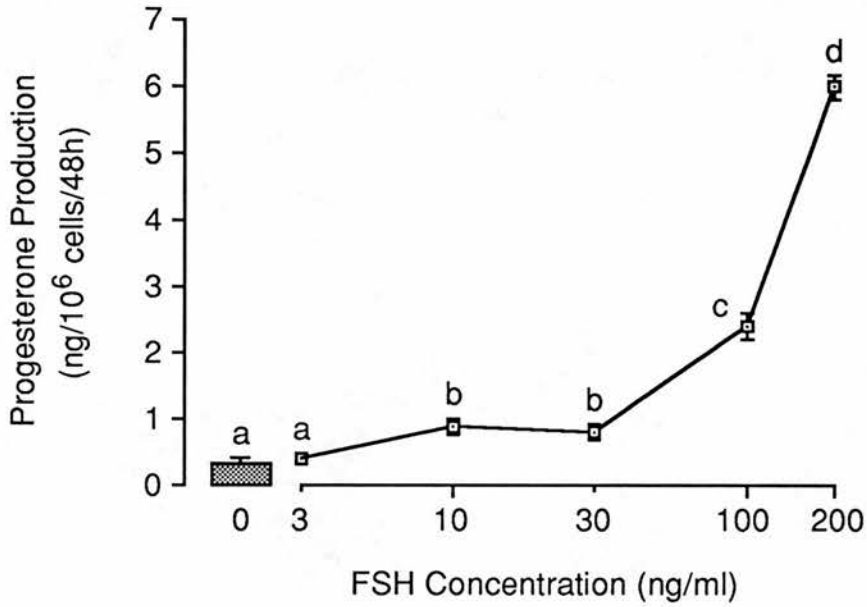


Fig. 2.3 Progesterone production by rat granulosa cells cultured in the presence or absence of increasing concentrations of hFSH. Values represent the mean (±SE) from triplicate wells. Points with different superscripts are significantly different from one another ($p<0.05$).

secretion by these cells, and at high concentrations of FSH (>30ng/ml), progesterone production was stimulated, but did not reach a plateau.

The effects of different sex steroids on basal and FSH-responsive progesterone production were investigated using cells from DES-treated animals. Figure 2.4 shows that, in the absence of FSH, oestradiol, 2-hydroxyoestradiol, testosterone and 5α-dihydrotestosterone had little or no effect on basal progesterone production by immature granulosa cells *in vitro*, across a wide dose-range. However, Figure 2.5 shows that, in the presence of a high dose (100ng/ml) of FSH, all four steroids (at a dose of 10^{-6}M) markedly augmented the stimulation of granulosa cell progesterone secretion by FSH, with an order of potency of E_2<2-OH-E_2<DHT<T.

Figure 2.6 shows a dose-response curve to FSH in the presence of 10^{-6}M testosterone, using the same range of FSH concentrations as in Fig. 2.3 (note that the scale is much larger than that of Fig. 2.3). In the presence of testosterone, the FSH dose-response curve was shifted to the left, with a minimally effective dose of FSH of <10ng/ml; maximal stimulation of progesterone synthesis being achieved at a dose of <100ng/ml, and a half maximal response at about 30ng/ml.

In the presence of FSH, all four steroids tested stimulated progesterone production throughout the range of concentrations used (Figs. 2.7 and 2.8). Indeed, at a plating density of $2x10^5$ cells per well, maximal augmentation of FSH-stimulated progesterone production may not have been reached even at steroid concentrations of 10^{-5}M. Concentrations of steroids greater than 10^{-5}M could not be used due to the low solubility of steroids in aqueous solution.

From these studies, it was concluded that a maximal dose of FSH was 100ng/ml, and that a dose of 10^{-6}M steroid was capable of significant augmentation of the stimulatory effect of FSH on granulosa cell progesterone production. This combination was used as the stimulus for studies of protein synthesis and for the optimisation of the culture conditions.

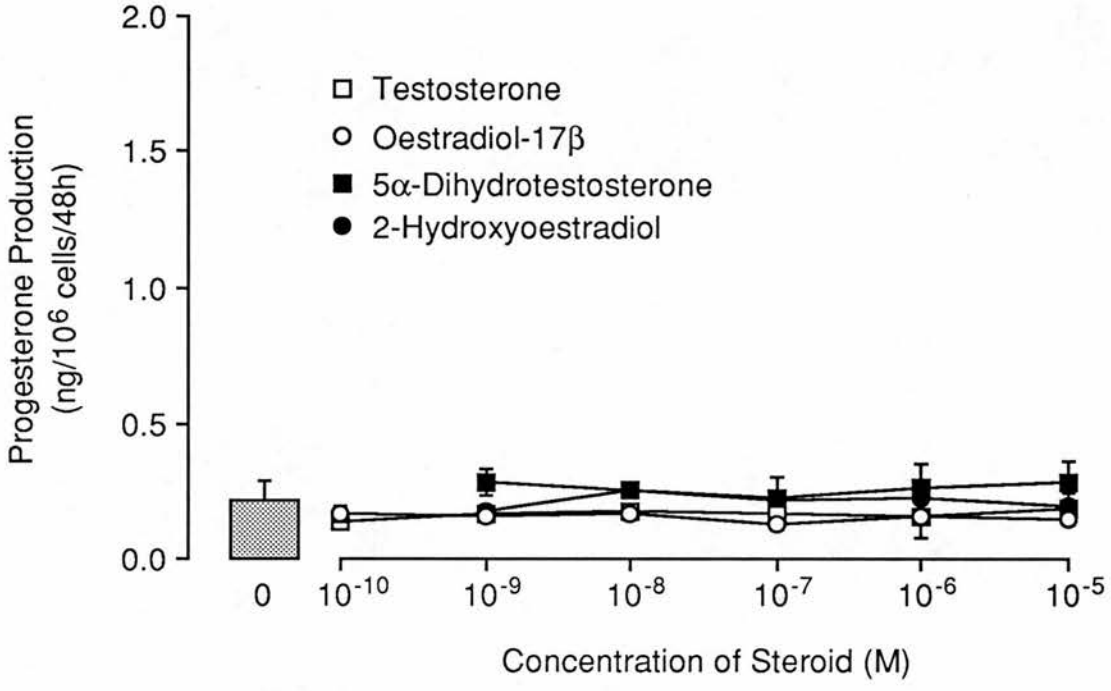


Fig. 2.4 Progesterone production by rat granulosa cells cultured in the presence or absence of increasing concentrations of oestradiol-17β, 2-hydroxyoestradiol, 5α-dihydrotestosterone or testosterone, in the absence of gonadotrophins. Values represent the mean (±SE) from triplicate wells. No significant differences were found between points.

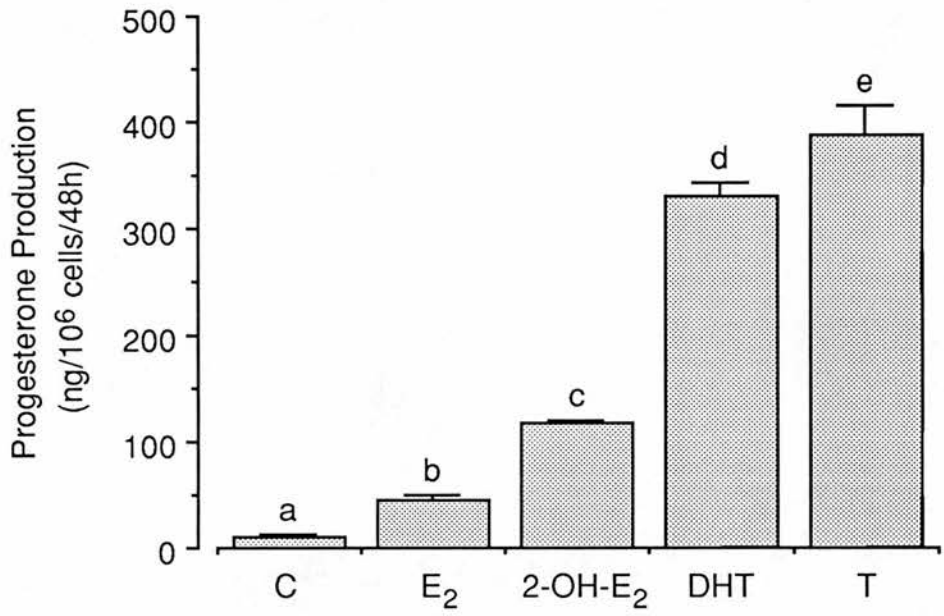


Fig.2.5 Progesterone production by rat granulosa cells cultured in the presence of 100ng/ml hFSH, and in the absence (C) or presence of 10^{-6}M oestradiol-17β (E_2), 2-hydroxyoestradiol (2-OH-E_2), 5α-dihydrotestosterone (DHT) or testosterone (T). Values represent the mean (±SE) from three separate experiments. Histograms with different superscripts are significantly different from one another ($p<0.05$).

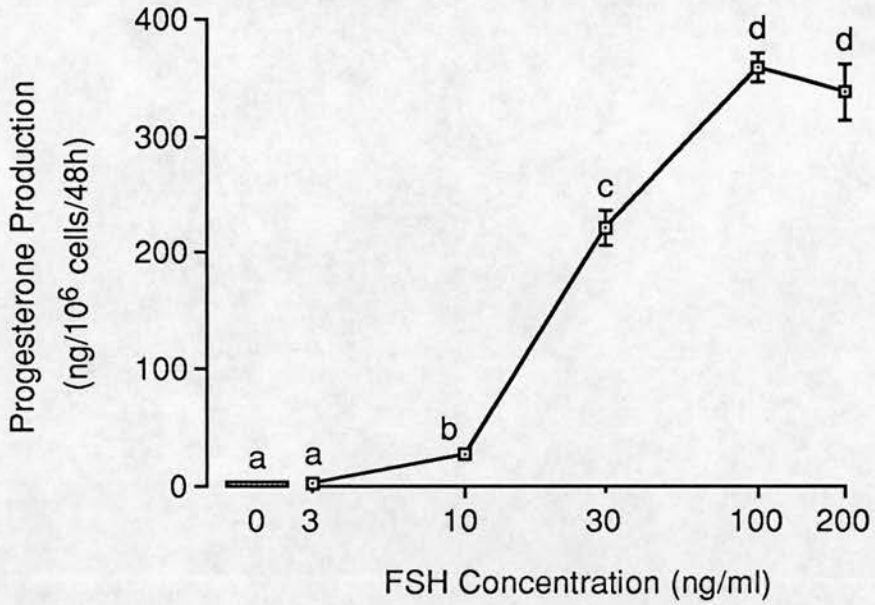


Fig. 2.6 Progesterone production by rat granulosa cells in the presence of 10^{-6}M testosterone and in the presence or absence of increasing concentrations of hFSH. Values represent the mean (±SE) from triplicate wells. Points with different superscripts are significantly different from one another ($p<0.05$).

Fig. 2.7 Progesterone production by rat granulosa cells cultured in the presence of 100ng/ml hFSH and in the presence or absence of increasing concentrations of oestradiol (A), or 2-hydroxyoestradiol (B). Values represent the mean (±SE) from six wells, from two separate experiments (A), or triplicate wells (B). In each graph, points with different superscripts are significantly different from one another ($p<0.05$).

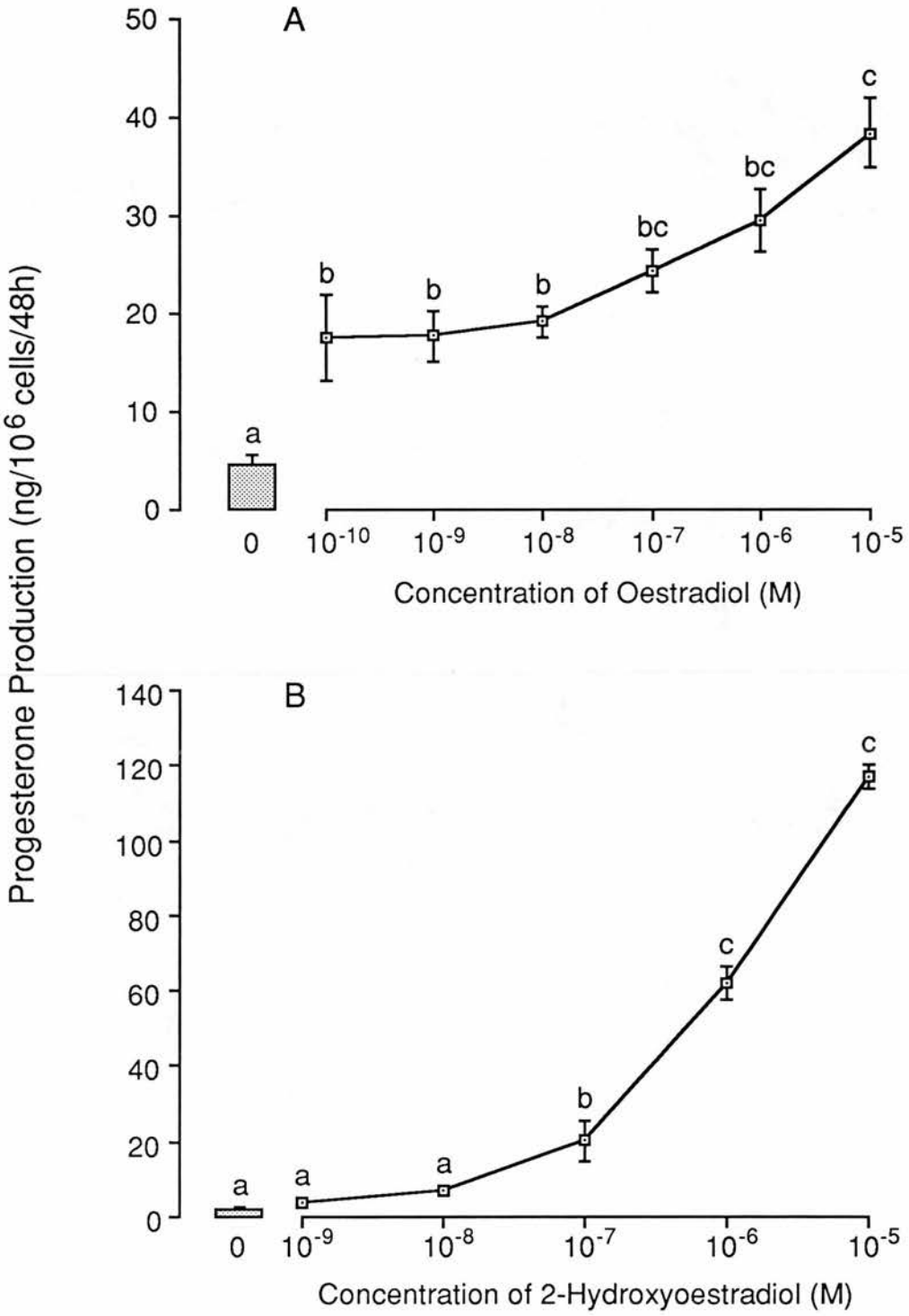
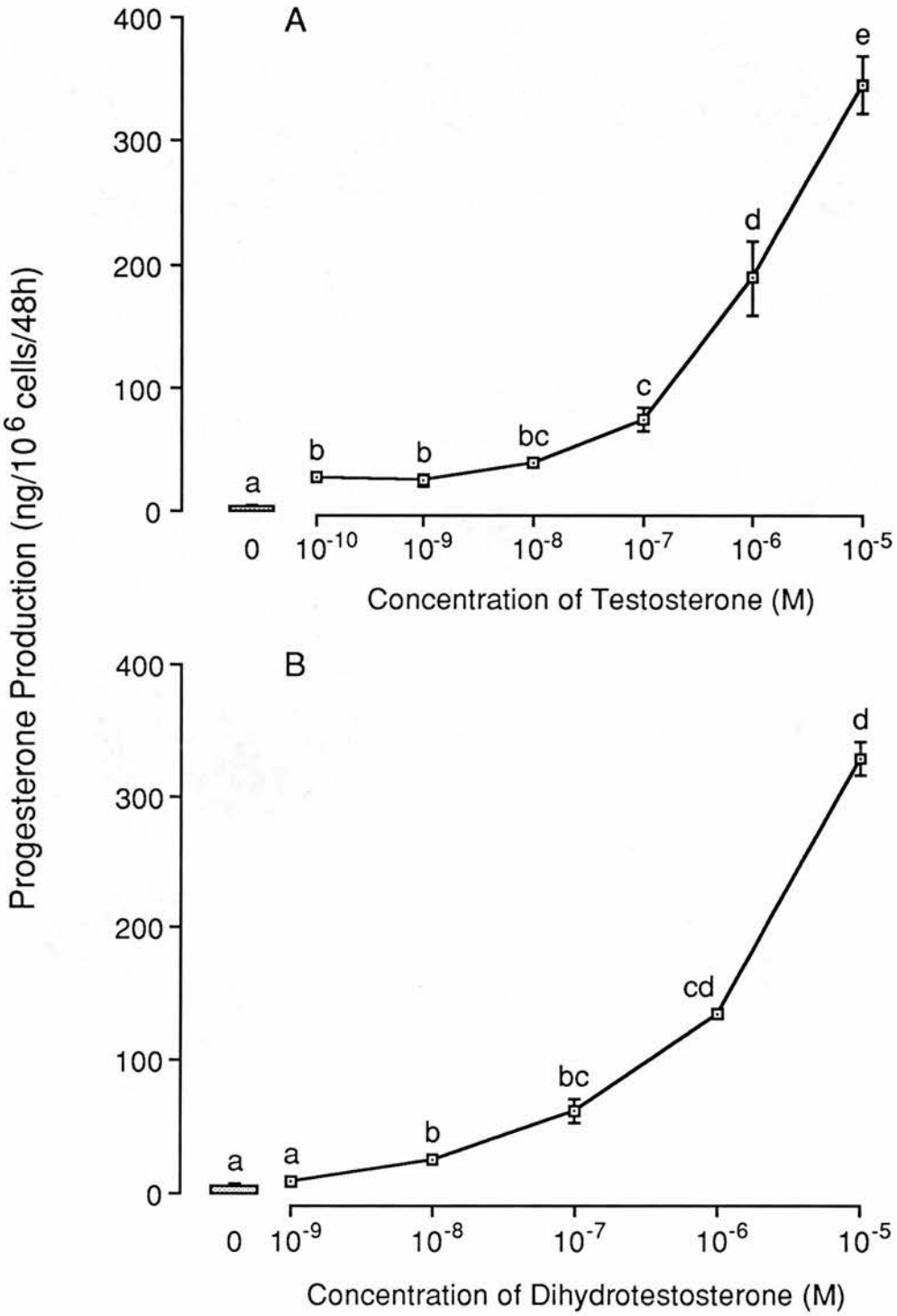

A
B
Progesterone Production (ng/10^6 cells/48h)
Concentration of Oestradiol (M)
Concentration of 2-Hydroxyoestradiol (M)
0
10^-10
10^-9
10^-8
10^-7
10^-6
10^-5
a
b
bc
c

Fig. 2.8 Progesterone production by rat granulosa cells cultured in the presence of 100ng/ml hFSH and in the presence or absence of increasing concentrations of testosterone (A), or 5α-dihydrotestosterone (B). Values represent the mean (±SE) from six wells, from two separate experiments (A), or triplicate wells (B). In each graph, points with different superscripts are significantly different from one another ($p<0.05$).


A
B
Progesterone Production (ng/10 6 cells/48h)
400
300
200
100
0
a
b
b
bc
c
d
e
0
10-10
10-9
10-8
10-7
10-6
10-5
Concentration of Testosterone (M)
a
a
b
bc
cd
d
10-9
10-8
10-7
10-6
10-5
Concentration of Dihydrotestosterone (M)

3.1.2 Protein Synthesis

The dramatic changes in patterns of secreted proteins induced by treatment of animals *in vivo* with FSH are shown by two dimensional electrophoresis in Fig. 2.9. Most strikingly, FSH treatment *in vivo* led to increased synthesis of a major neutral protein of approximately 24kDa molecular weight. Synthesis of several other abundant proteins was increased by FSH treatment *in vivo*, including three neutral proteins with molecular weights between 30 and 35kDa, and two others migrating at approximately 70 and 80kDa. An acidic ~27kDa minor protein was also induced by FSH. Also, synthesis of at least four proteins was reduced by FSH treatment, the most abundant being a ~40kDa neutral protein and a ~55kDa acidic protein.

This treatment also induced changes in cellular proteins, shown in Fig. 2.10 by one dimensional SDS-PAGE. The most prominent changes in cellular protein synthesis were the reduced synthesis of proteins with approximate molecular weights of 45kDa and 35kDa, and the increased synthesis of a 24kDa protein.

The *in vitro* effects of FSH and steroids on patterns of cellular protein synthesis by granulosa cells are shown in Fig. 2.11A. FSH alone caused a slight increase in the synthesis of two moderately abundant proteins of approximately 65 and 24kDa, and a slight decrease in the synthesis of two other proteins about 100 and 32kDa in size. The effect of FSH on these proteins was enhanced by simultaneous treatment with 10^{-6}M T, E_2 or 2-OH-E_2, especially with regard to the increase in synthesis of the 24kDa protein. Steroids had no discernible effect on patterns of protein synthesis in the absence of FSH. The effect of FSH and steroids on the metabolism of the 32kDa protein may have been an increase in its molecular weight rather than a decrease in its synthesis, since a band migrating at a slightly higher molecular weight appeared to become more diffuse as the 32kDa band was lost. Treatment with these steroids in the presence of FSH also revealed changes in the synthesis of two other proteins; synthesis of a 28kDa protein was slightly increased, and that of an 85kDa protein was slightly reduced. T appeared to be the most potent

Fig. 2.9 Two dimensional polyacrylamide electrophoresis of rat granulosa cell secreted proteins. Animals were treated only with DES for 4 days before isolation of granulosa cells (A) or injected with 20μg oFSH every 12 hours for 2 days in addition to DES treatment (B) before granulosa cell isolation. After isolation, cells were cultured for 24h in methionine-free MEM containing ^{35}S-labelled methionine to label granulosa cell proteins. Equal amounts ($1x10^5$ cpm) of TCA precipitable radiolabelled proteins secreted into the culture medium were subjected to isoelectric focussing and SDS-PAGE. Gels (12.5% polyacrylamide) were soaked in Amplify, dried and exposed to X-ray film for 2 days at -70C. Migration of molecular weight standards are marked on the left, and migration of standards of known isoelectric points are marked along the bottom of each gel. Proteins whose abundance decreased with FSH treatment are circled in A, and those whose abundance increased with FSH treatment are circled in B.

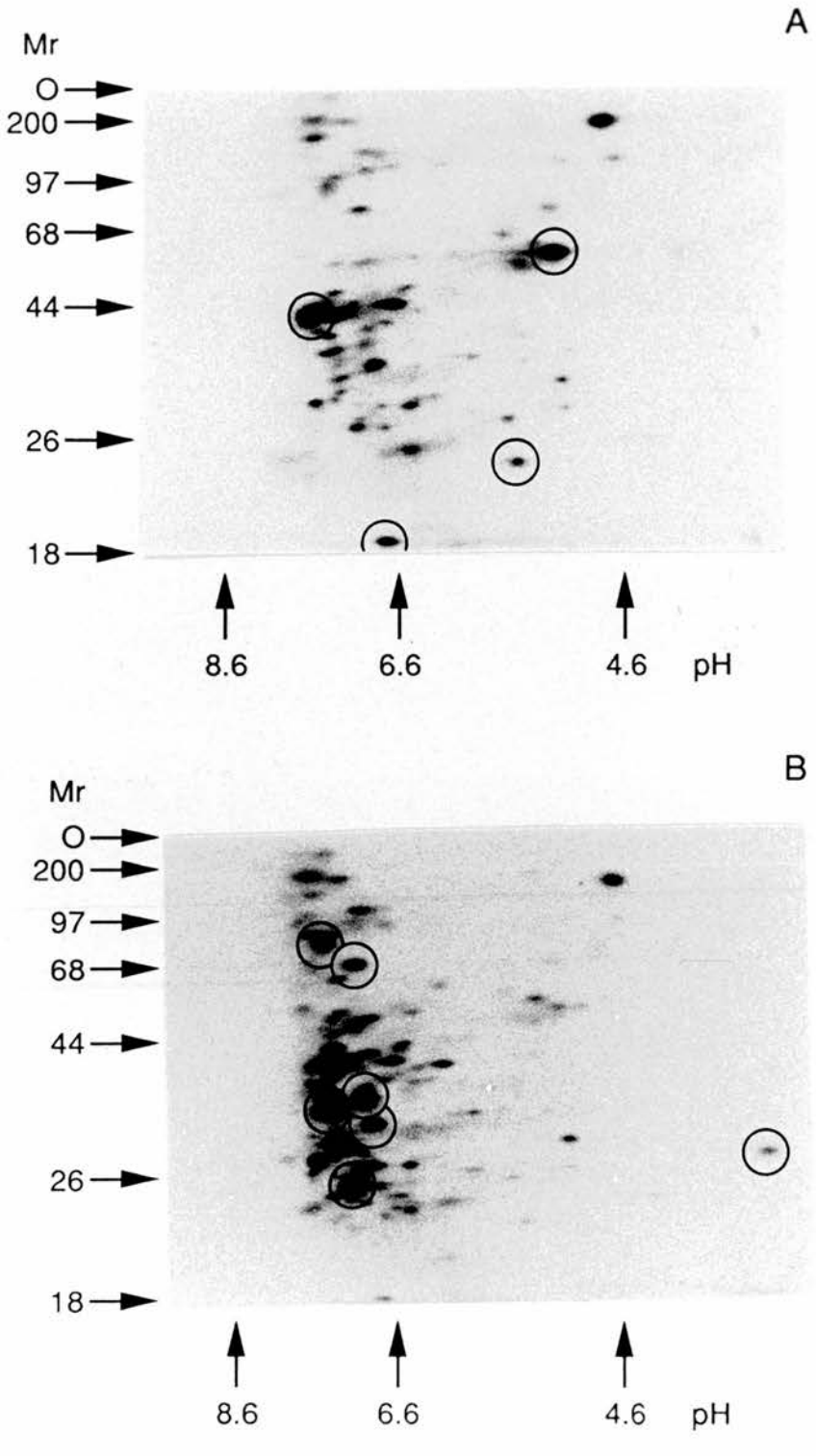

A
Mr
O
200
97
68
44
26
18
8.6
6.6
4.6
pH
B
Mr
O
200
97
68
44
26
18
8.6
6.6
4.6
pH

Fig. 2.10 One dimensional SDS-PAGE of granulosa cell cellular proteins. Animals were treated and cells cultured as described in the legend to Fig. 2.9. Radiolabelled cellular proteins were obtained by lysis of cell monolayers with detergent, and equal amounts of TCA precipitable radiolabelled protein ($4x10^4$ cpm) were separated by SDS-PAGE in 7.5-15% polyacrylamide gradient gels. Gels were dried and exposed to X-ray film for 3 days at room temperature.

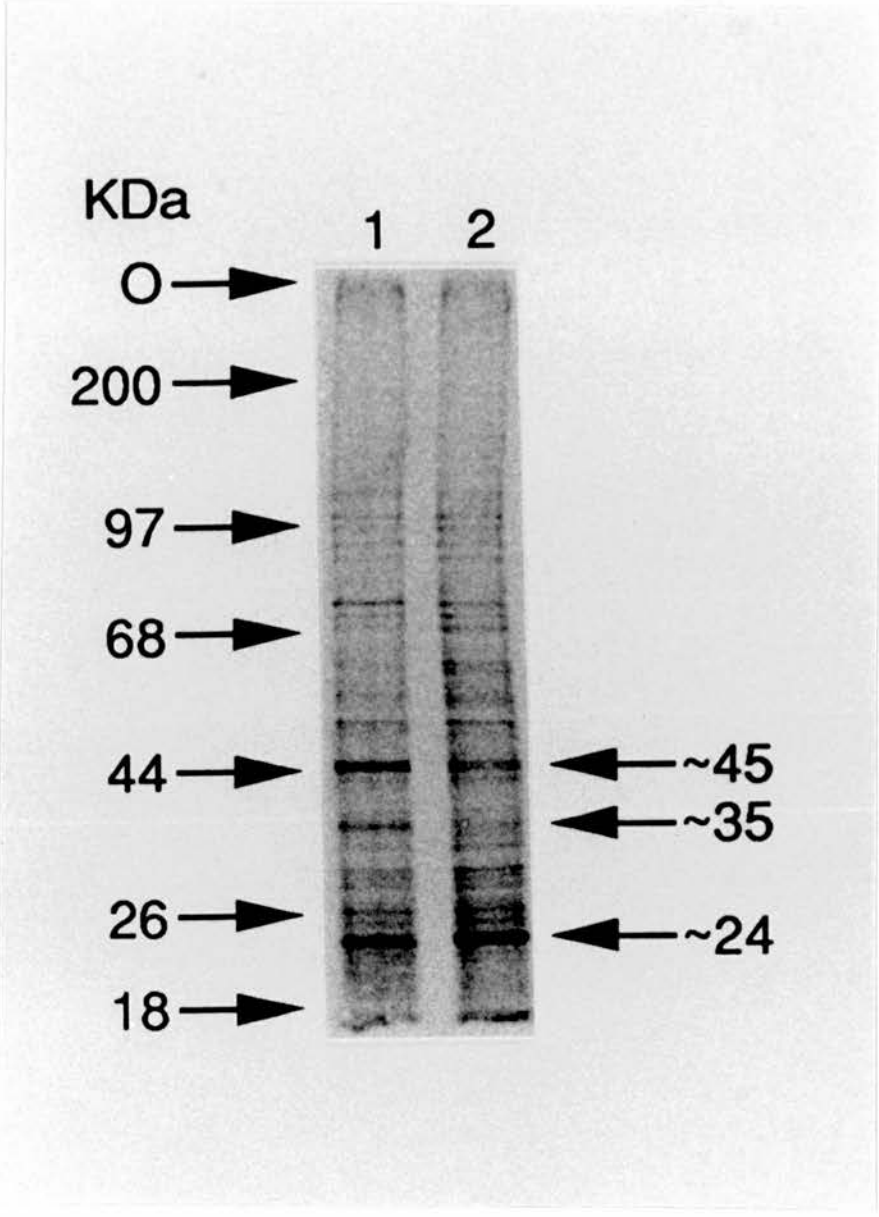
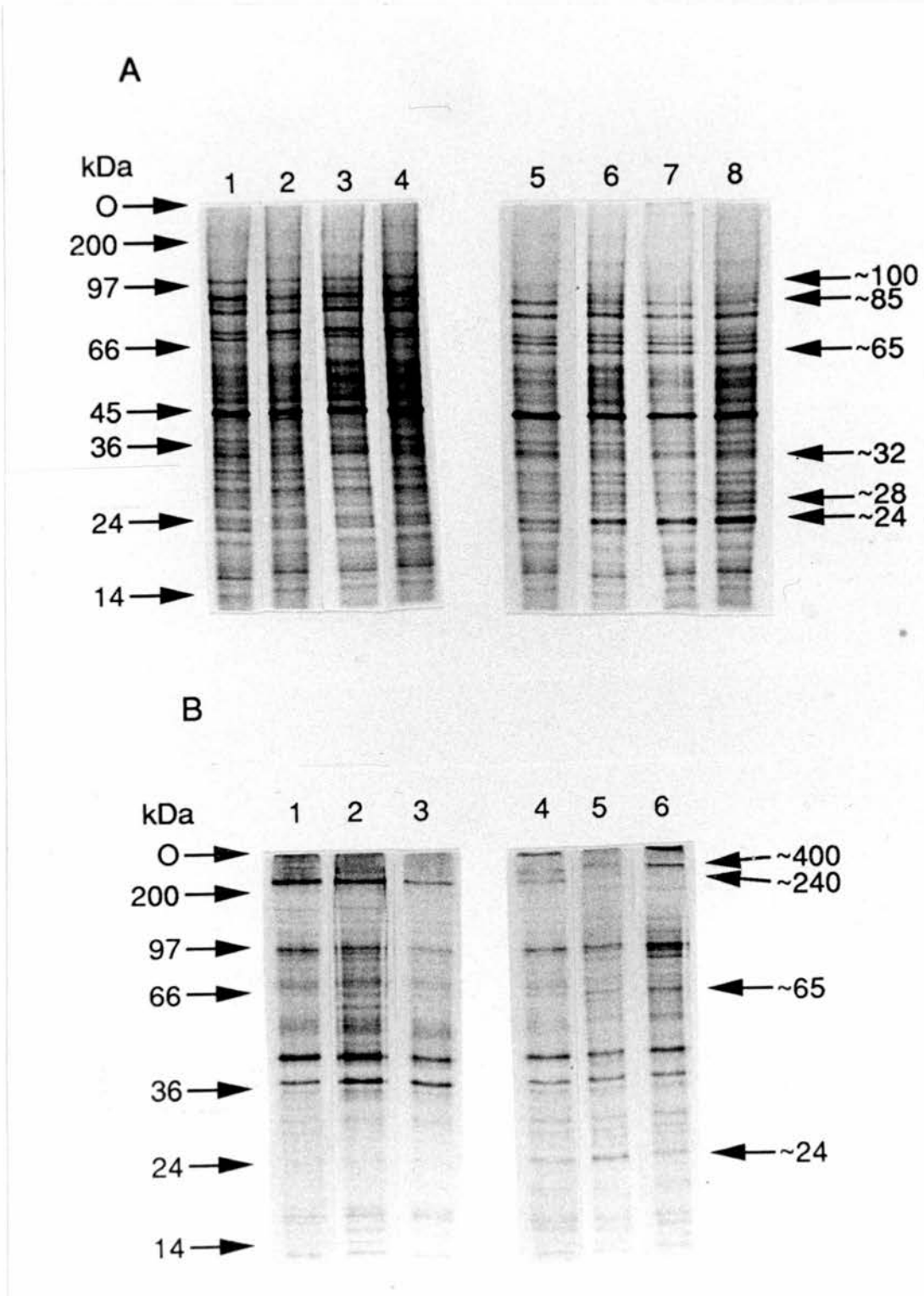

KDa
1
2
O
200
97
68
44
26
18
~45
~35
~24

Fig. 2.11 One dimensional SDS-PAGE of cultured granulosa cell cellular and secreted proteins. Cells were incubated for 48h in the presence or absence of hFSH and/or steroids. Equal amounts ($2x10^4$ cpm) of radiolabelled protein from each sample were subjected to electrophoresis in 7.5-12.5% polyacrylamide gels. Gels were dried and exposed to x-ray film for 7 days at room temperature. Panel A. Cellular proteins: Lane 1. Control. Lane 2. 10^{-6}M oestradiol alone. Lane 3. 10^{-6}M 2-hydroxyoestradiol alone. Lane 4. 10^{-6}M testosterone alone. Lane 5. 100ng/ml hFSH alone. Lane 6. 100ng/ml hFSH and 10^{-6}M oestradiol. Lane 7. 100ng/ml hFSH and 10^{-6}M 2-hydroxyoestradiol. Lane 8. 100ng/ml hFSH and 10^{-6}M testosterone. Panel B. Secreted proteins: Lane 1. Control. Lane 2. 10^{-6}M oestradiol alone. Lane 3. 10^{-6}M testosterone alone. Lane 4. 100ng/ml hFSH alone. Lane 5. 100ng/ml hFSH and 10^{-6}M oestradiol. Lane 6. 100ng/ml hFSH and 10^{-6}M testosterone.


A
kDa
1 2 3 4 5 6 7 8
O
200
97
66
45
36
24
14
~100
~85
~65
~32
~28
~24
B
kDa
1 2 3 4 5 6
O
200
97
66
36
24
14
~400
~240
~65
~24

of the three steroids, but because of small differences in the loading of radiolabelled proteins on the lanes of the gels, it was not possible to determine the order of potency.

Patterns of protein secretion were also influenced by FSH and steroids. Fig. 2.11B shows one dimensional SDS-PAGE of radiolabelled proteins secreted into the culture medium by granulosa cells after 48h incubation with FSH and steroids. The most striking effects were a decrease in the synthesis and secretion of a large major protein with a molecular weight of approximately 240kDa, and an increase in the synthesis of a protein migrating at the same molecular weight as the 24kDa cellular protein, the synthesis of which was also stimulated by FSH. The suppression by FSH of the synthesis of the 240kDa protein was markedly augmented by steroids. The synthesis of at least two other proteins appeared to be stimulated by FSH, one very large with a molecular weight of about 400kDa, and one about 65kDa molecular weight.

3.2 Time-Course of Responses

3.2.1 Progesterone Production

Fig. 2.12 shows progesterone accumulation in the culture medium at 6, 24, 48 and 72h after plating of cells in medium containing 100ng/ml FSH and 10^{-6}M T. It can be seen that the accumulation of progesterone increased with time in culture throughout the duration of the experiment, most of the progesterone being produced during the second day in culture.

3.2.2 Protein Synthesis

Fig. 2.13 shows one dimensional SDS-PAGE gels of cellular proteins synthesised during the labelling period by cells preincubated for different times in the presence or absence of 100ng/ml FSH and 10^{-6}M T. A protein migrating at approximately 24kDa was present in abundance in the monolayers of freshly isolated cells and those cells which had been

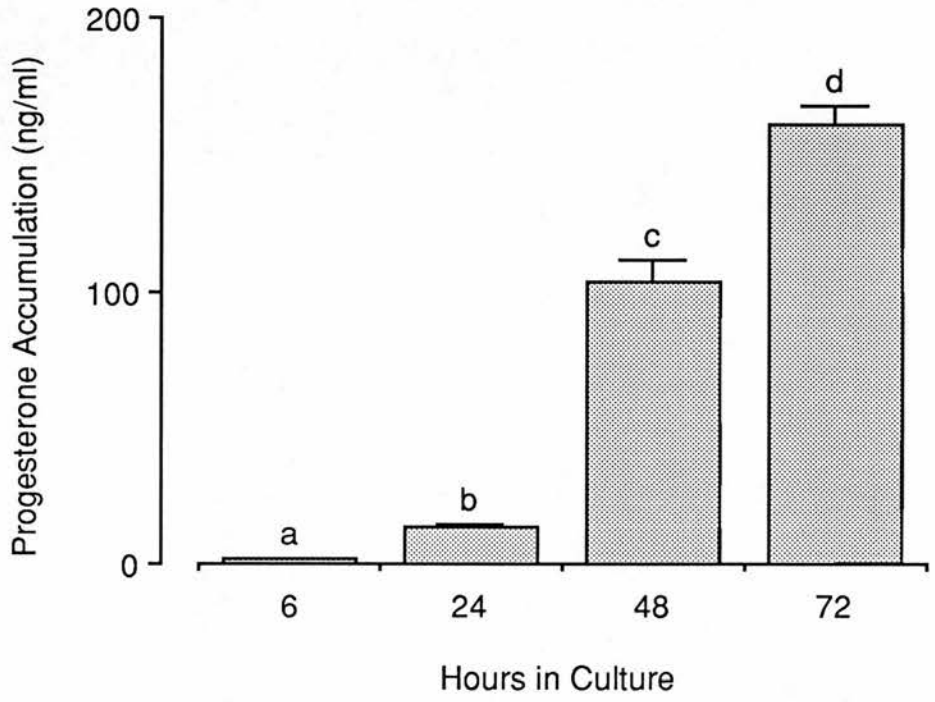
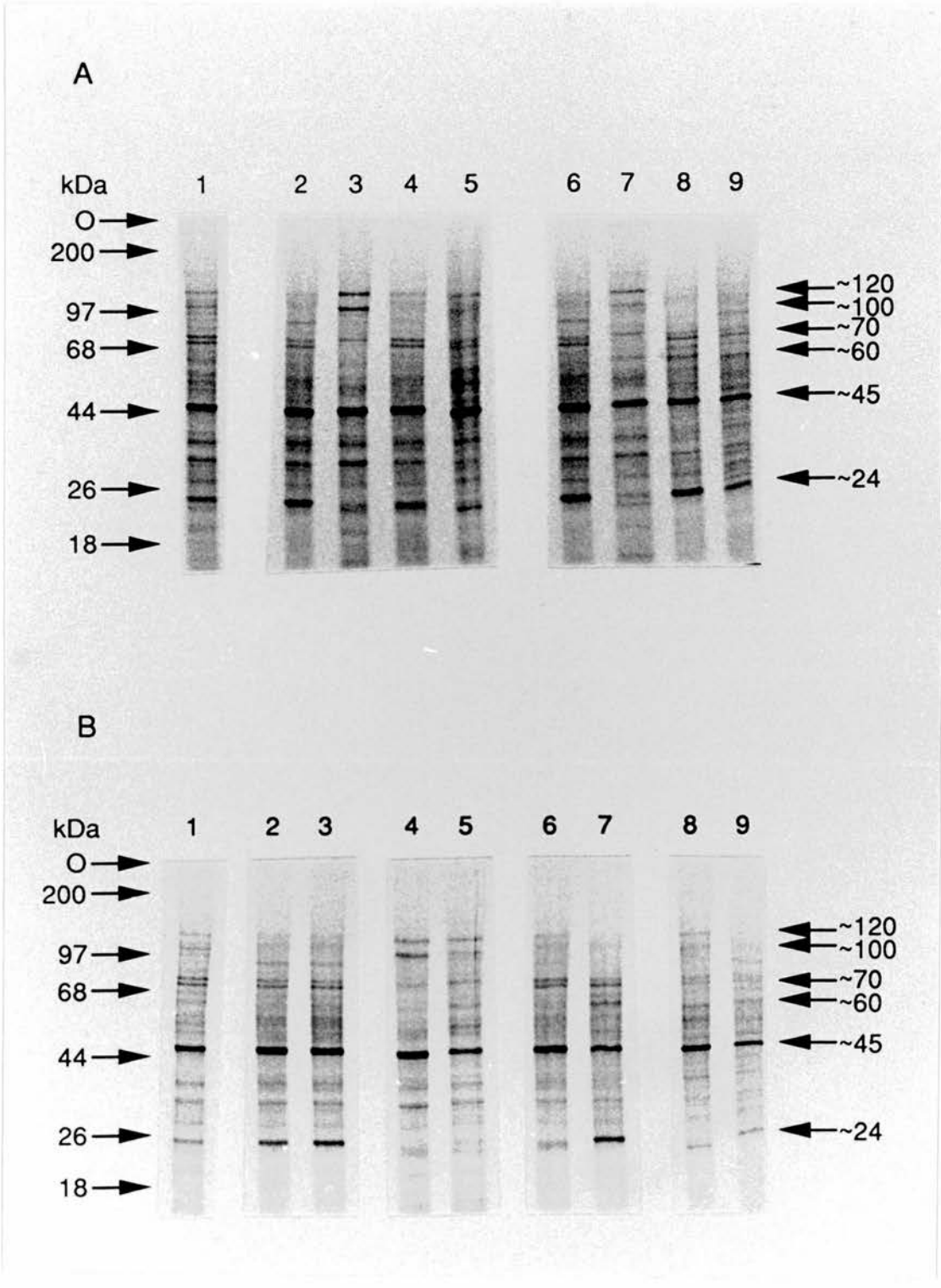


Fig. 2.12 Progesterone accumulation in the medium from rat granulosa cells cultured for 6, 24, 48 or 72h, in the presence of 100ng/ml hFSH and 10^{-6}M testosterone. Values represent the mean (±SE) from triplicate wells. Histograms with different superscripts are significantly different from one another ($p<0.05$).

Fig.2.13 One dimensional SDS-PAGE of metabolically labelled cellular proteins synthesised by rat granulosa cells cultured in the presence or absence of 100ng/ml hFSH and 10^{-6}M testosterone for different times before the labelling period. Equal amounts ($2x10^4$ cpm) of radiolabelled protein from each sample were subjected to electrophoresis in 7.5-12.5% polyacrylamide gels. Gels were dried and exposed to X-ray film for 4 days at room temperature. Top panel (A); Lane 1, no preincubation. Lanes 2-5, control. Lanes 6-9, FSH and testosterone. Lanes 2 and 6, 6h preincubation. Lanes 3 and 7, 24h preincubation. Lanes 4 and 8, 48h preincubation. Lanes 5 and 9, 72h preincubation. The same samples are also shown in the lower panel (B) in the following order; Lane 1, no preincubation. Lanes 2, 4, 6 and 8, control. Lanes 3, 5, 7 and 9, FSH plus testosterone. Lanes 2 and 3, 6h preincubation. Lanes 4 and 5, 24h preincubation. Lanes 6 and 7, 48h preincubation. Lanes 8 and 9, 72h preincubation.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B
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6
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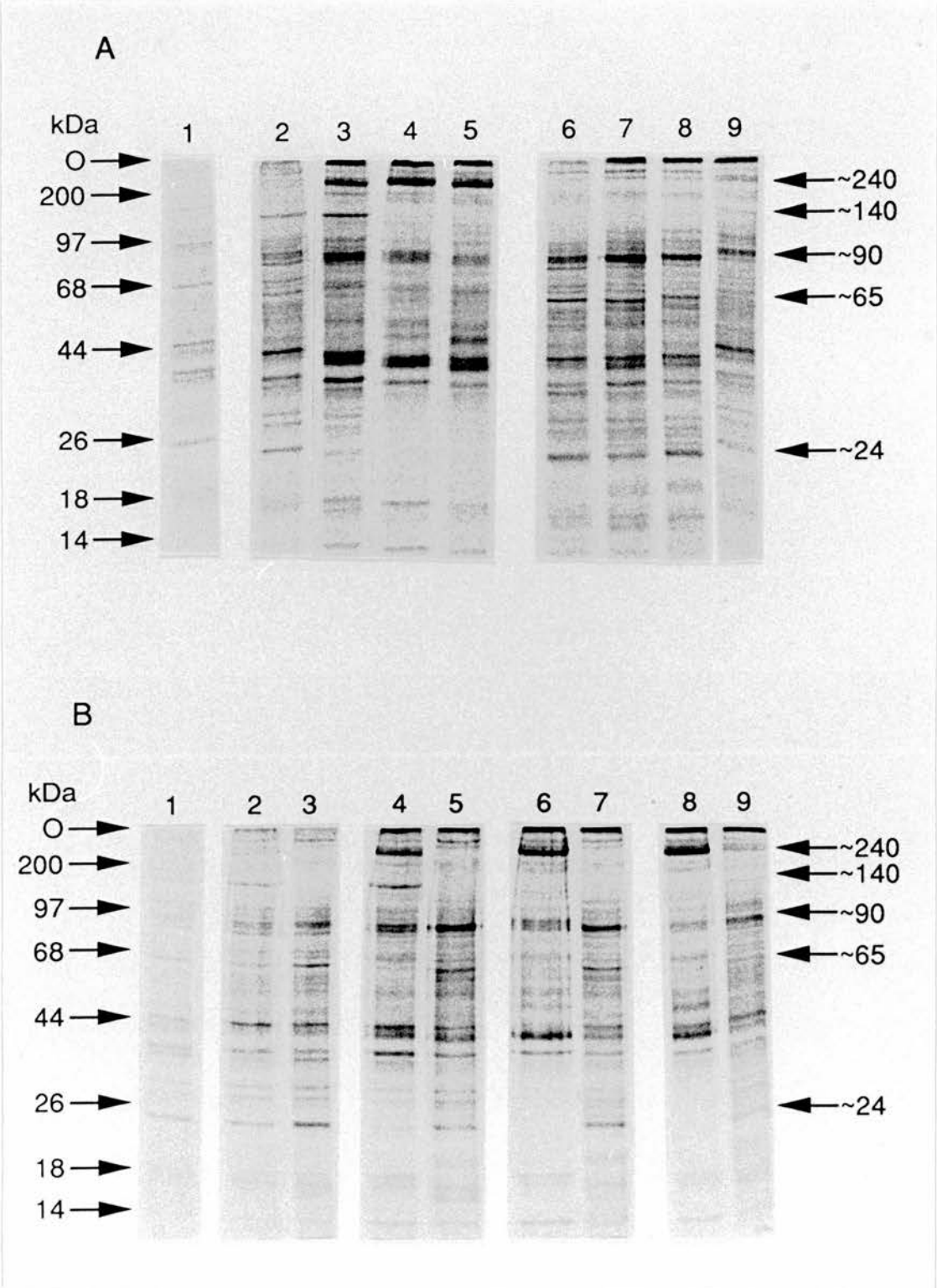
9
O
200
97
68
44
26
18
~120
~100
~70
~60
~45
~24

preincubated for 6h, and was synthesised to a lesser degree by cells incubated for longer periods. Consequently, after 6h preincubation, the previously observed stimulation by FSH of the synthesis of this protein (see Fig. 2.11A) was not seen. After 24h, the pattern of protein synthesis was markedly different from the patterns observed at other time-points, a major protein of approximately 100kDa molecular weight being induced, synthesis of a ~120kDa protein being enhanced, and a less abundant ~70kDa protein being lost. Moreover, no effect of FSH and T on the synthesis of the ~24kDa protein by these cells was observed. At this time point, synthesis of a ~60kDa protein was slightly increased, and that of a ~45kDa protein was reduced by FSH and T treatment. After 48h preincubation, a strong induction of the ~24kDa protein by FSH and T was seen. The stimulation of synthesis of the ~60kDa protein, and inhibition of synthesis of the ~45kDa protein by FSH and T were more marked after 48h preincubation. The pattern of cellular protein synthesis and the response to FSH and T after 72h preincubation were similar to those at 48h, although less pronounced.

The patterns of secreted protein synthesis under the same conditions are shown in Fig. 2.14. The prominent ~240kDa FSH-responsive protein secreted by untreated cells (see Fig. 2.11B) did not appear until cells had been preincubated for at least 24h. Conversely, the ~24kDa protein observed in the medium from FSH-treated cells (see Fig. 2.11B) was secreted by freshly isolated cells and untreated cells which had been preincubated for 6 or 24h, and was lost after more prolonged incubation of untreated cells. Because of the lack of secretion of the ~240kDa protein after 6h preincubation and the elevated secretion of the ~24kDa protein after 6 or 24h preincubation by untreated cells, no effect of FSH and T on the secretion of these two proteins was observed unless cells had been preincubated for at least 48h. The effect of FSH and T after 72h of pretreatment was less marked than after 48h.

Fig.2.14. One dimensional SDS-PAGE of metabolically labelled secreted proteins synthesised by rat granulosa cells cultured in the presence or absence of 100ng/ml hFSH and 10^{-6}M testosterone for different times before the labelling period. Equal amounts ($2x10^4$ cpm) of radiolabelled protein from each sample was subjected to electrophoresis in 7.5-12.5% polyacrylamide gels. Gels were dried and exposed to X-ray film for 6 days at room temperature. Top panel (A); Lane 1, no preincubation. Lanes 2-5, control. Lanes 6-9, FSH and testosterone. Lanes 2 and 6, 6h preincubation. Lanes 3 and 7, 24h preincubation. Lanes 4 and 8, 48h preincubation. Lanes 5 and 9, 72h preincubation. The same samples are also shown in the lower panel (B), in the following order; Lane 1, no preincubation. Lanes 2, 4, 6 and 8, control. Lanes 3, 5, 7 and 9, FSH plus testosterone. Lanes 2 and 3, 6h preincubation. Lanes 4 and 5, 24h preincubation. Lanes 6 and 7, 48h preincubation. Lanes 8 and 9, 72h preincubation.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97
68
44
26
18
14
1
2
3
4
5
6
7
8
9
~240
~140
~90
~65
~24

3.3 Effect of Plating Density

3.3.1 Progesterone Production

Fig. 2.15 shows accumulation of progesterone in the culture medium 48h after treatment of cells plated at different densities with 100ng/ml FSH and 10^{-6}M T. Progesterone accumulation increased with cell numbers up to a density of $3x10^5$ cells per well, and then reached a plateau, suggesting that some hormonal responsiveness was lost at high cell density. After 48h of incubation, cell monolayers were counted in a haemocytometer as described above to ascertain the actual numbers of cells attached to the culture wells in each nominal cell density group. Fig. 2.16A shows that the number of cells observed after 48h of culture in the absence of hormonal treatment correlated well with the nominal plating density, indicating that little cell proliferation or death had occurred over that time. However, in groups where cells had been treated with FSH and T, the number of cells was consistently lower than in the corresponding control group. A further experiment was carried out, in which cell monolayers were not washed before being detached and cells counted. Under these conditions (Fig. 2.16B), the numbers of cells counted after 48h were the same in both groups.

3.3.2 Protein Synthesis

Fig. 2.17A shows the yield of TCA precipitable counts from cell monolayers cultured at different cell densities in the presence or absence of 100ng/ml FSH and 10^{-6}M T. As expected, the yield of radiolabelled protein increased with cell density throughout the range of densities studied. However, yield of radiolabelled protein (Fig. 2.17B) secreted into the medium decreased with increasing cell density. Moreover, yields were much lower from media than from cell monolayers (compare scales on y-axes of Figs. 2.17A and 2.17B).

Metabolically labelled proteins were then subjected to one dimensional SDS-PAGE to investigate possible effects of cell density on

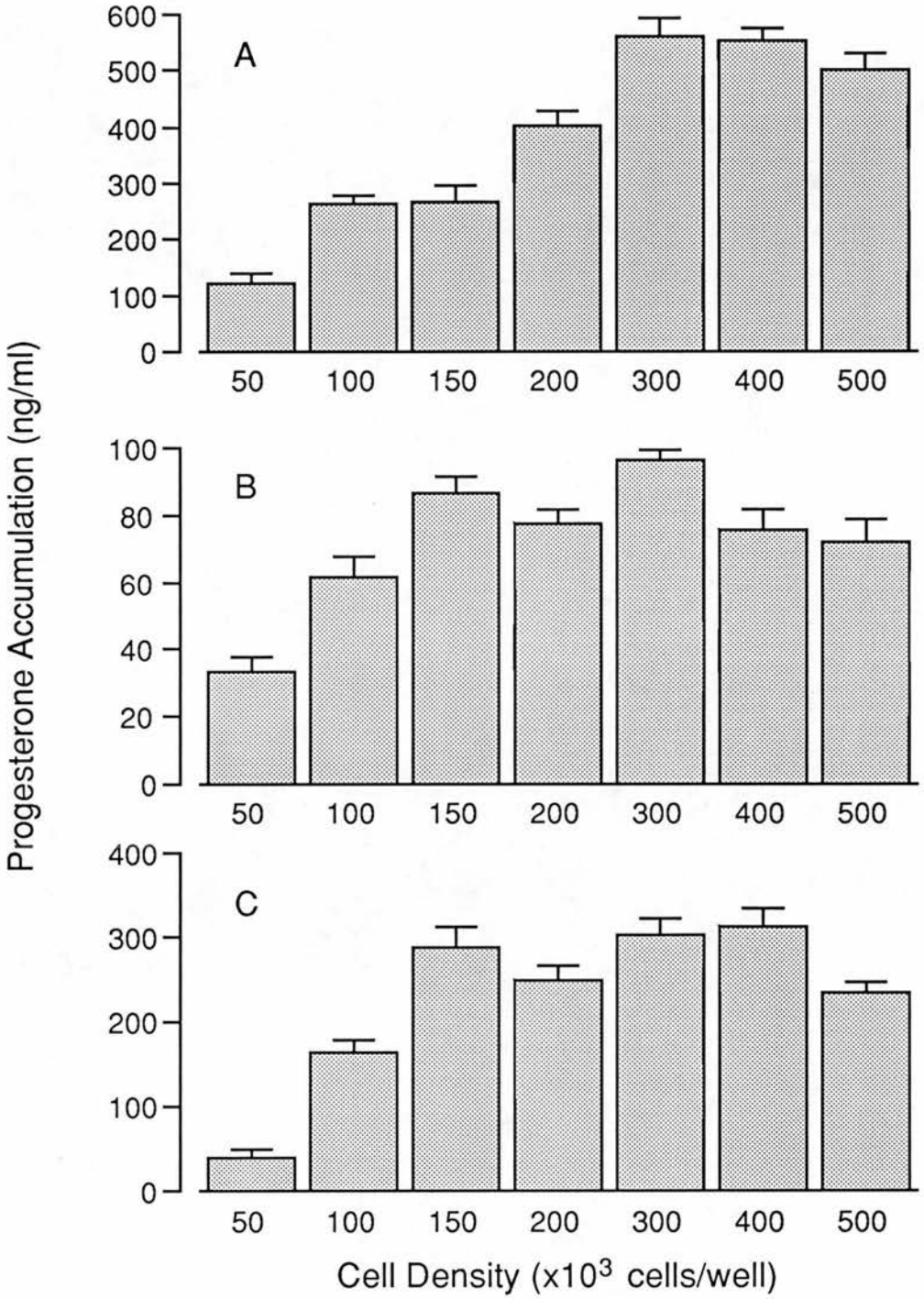


Fig.2.15. Progesterone production by rat granulosa cells cultured at different plating densities. Cells were stimulated with 100ng/ml hFSH plus 10^{-6}M testosterone (A), oestradiol-17β (B) or 2-hydroxyoestradiol (C). Values represent the mean (±SE) from triplicate wells.

Fig.2.16. Numbers of cells attached to culture plates after 48h incubation at different cell densities in the presence or absence of 100ng/ml hFSH plus 10^{-6}M testosterone. A. Cell monolayers were washed once with 1ml MEM before counting. B. Cells were not washed before counting. Cells were recovered from the wells and counted as described in section 2.3. Values represent the mean (±SE) from counts from triplicate wells, counting 4 fields of a haemocytometer for each well. Asterisks in A denote statistically significant differences between control and treated at individual cell densities ($p<0.05$). In B, no significant differences in cell numbers were found between control and treated at any cell density.

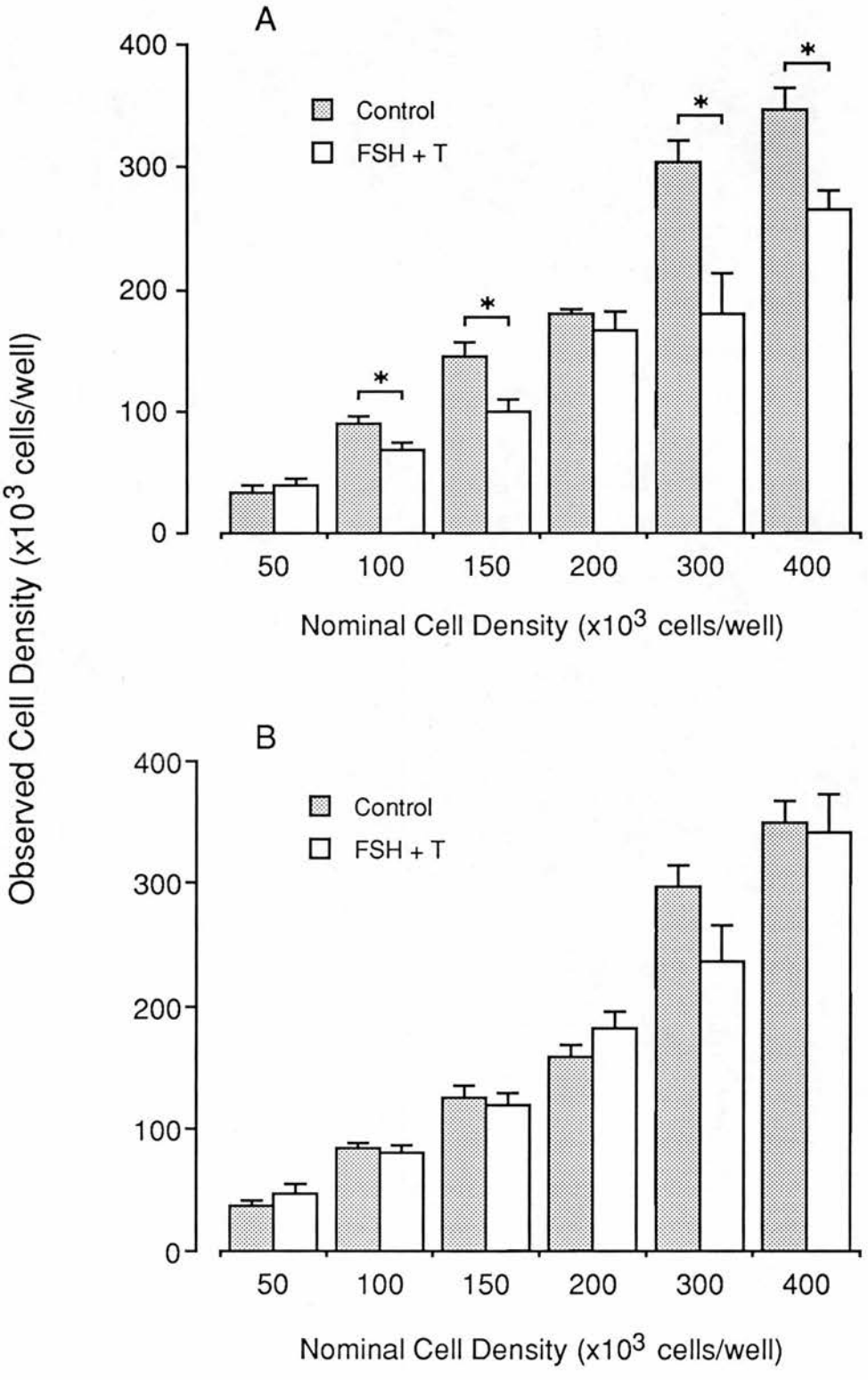
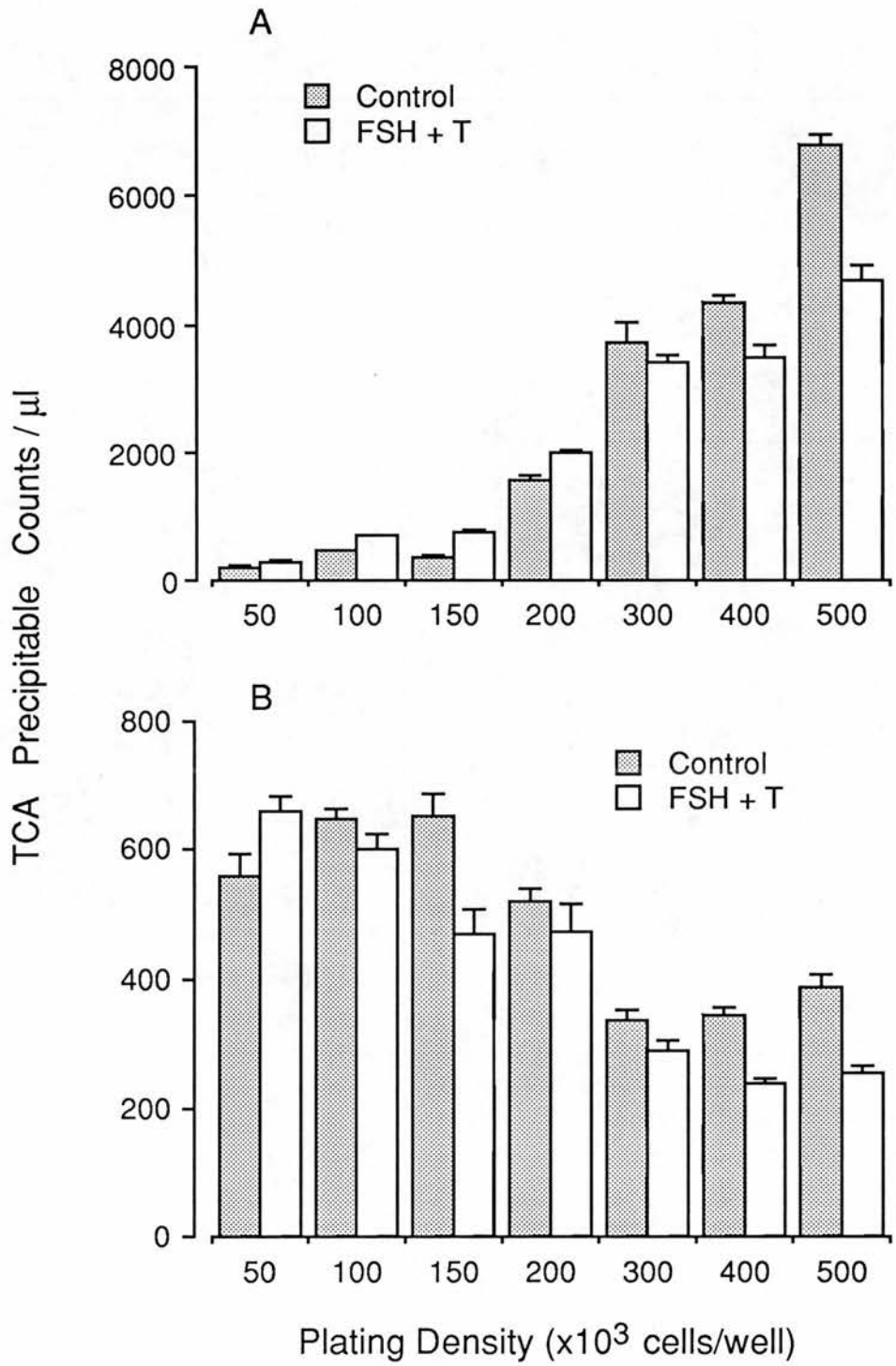

A
400
300
200
100
0
Control
FSH + T
*
*
*
50
100
150
200
300
400
Nominal Cell Density (x10^3 cells/well)
B
400
300
200
100
0
Control
FSH + T
50
100
150
200
300
400
Nominal Cell Density (x10^3 cells/well)
Observed Cell Density (x10^3 cells/well)

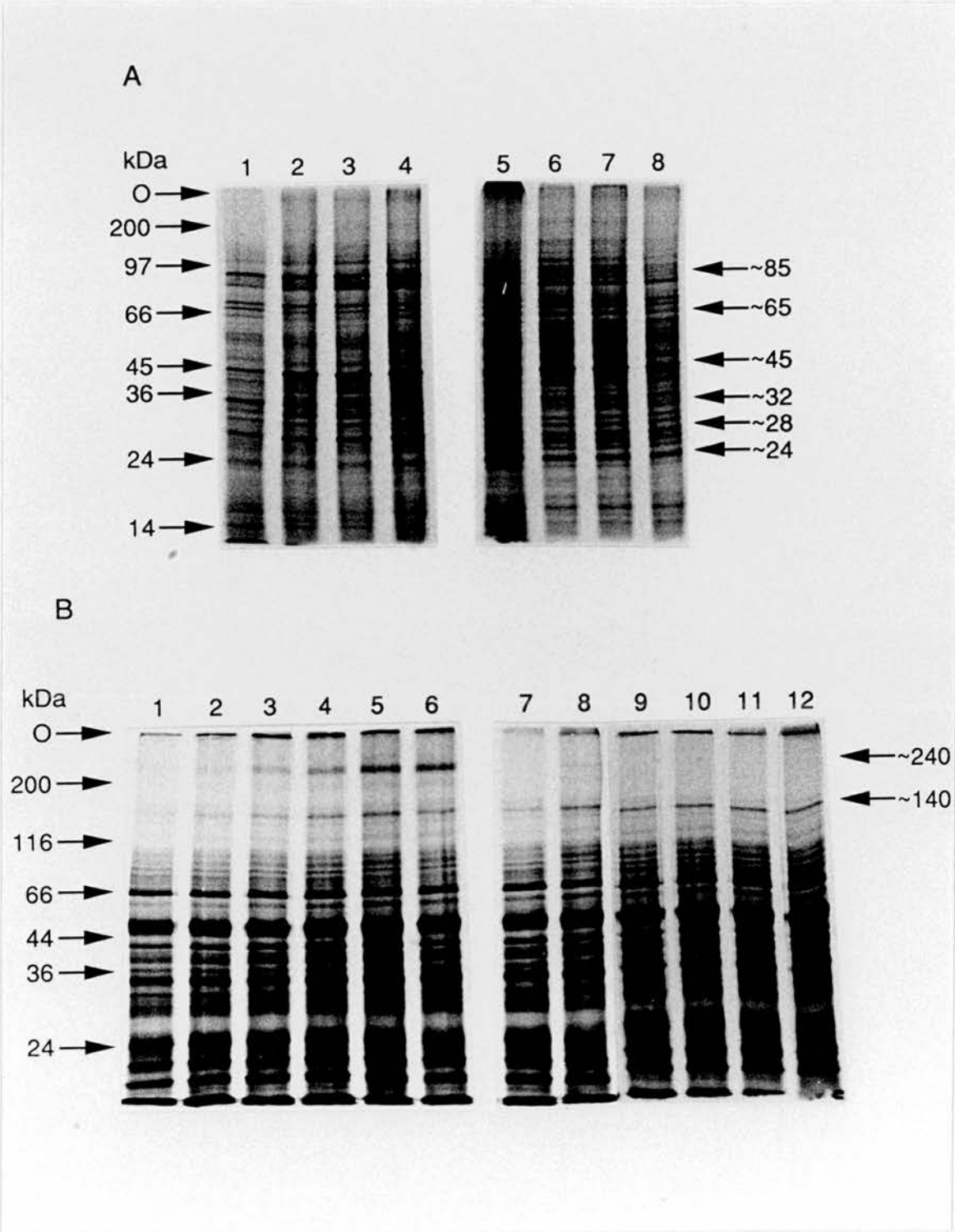
Fig.2.17. Trichloroacetic acid precipitable counts from radiolabelled protein samples from rat granulosa cells cultured at different plating densities, in the presence (open bars) or absence (shaded bars) of 100ng/ml hFSH plus 10^{-6}M testosterone; A) cellular proteins, and B) secreted proteins. Values represent the mean (±SE) from six wells, from two separate experiments.


A
8000
6000
4000
2000
0
Control
FSH + T
50
100
150
200
300
400
500
B
800
600
400
200
0
Control
FSH + T
50
100
150
200
300
400
500
TCA Precipitable Counts / µl
Plating Density (x10³ cells/well)

the patterns of protein synthesis and the changes in patterns induced by FSH and testosterone. Fig. 2.18A shows cellular proteins extracted from cell monolayers seeded at different densities and cultured for 48h in the presence or absence of 100ng/ml FSH and 10^{-6}M T. The only effect of cell density on the patterns of protein synthesis observed was a reduced synthesis of a prominent ~45kDa protein by FSH-treated cells at high cell density ($4x10^5$ cells/well). The most prominent effects of FSH and T were the suppression of the synthesis of the ~85 and ~32kDa proteins, and the enhancement of synthesis of the ~24 and ~65kDa proteins. Synthesis of several other proteins with molecular weights between 25 and 35kDa was also enhanced by FSH treatment. At high cell density ($4x10^5$ cells/well), treatment with FSH and T caused a reduction in the synthesis of the ~45kDa protein (see above).

The SDS-PAGE gels on which the corresponding culture medium proteins were separated are shown in Fig. 2.18B. No effect of cell density on the pattern of protein secretion by FSH-treated cells was observed. However, the pattern of protein secretion by control cells was affected by cell density. Secretion of two proteins with approximate molecular weights of 240 and 140kDa increased with cell density. One of these proteins migrated at the same molecular weight as the ~240kDa protein the secretion of which was shown to be inhibited by FSH treatment. As above, the most striking effect of FSH and T was the inhibition of the synthesis and secretion of the 240kDa protein. Due to the effect of cell density on the basal secretion of this protein, this effect of FSH was most easily seen at high cell densities ($1.5x10^5$ or more cells/well). It should be noted that the overall pattern of protein secretion in this experiment was slightly different from that observed in some experiments such as that presented in Fig. 2.11B. In particular, the reproducible stimulation of secretion of the ~24kDa protein was not observed in this experiment.

Fig.2.18. Effect of plating density on patterns of protein synthesis and response to FSH and testosterone. $2x10^4$ cpm of radiolabelled protein from each sample was subjected to electrophoresis in 7.5-12.5% polyacrylamide gels. Gels were dried and exposed to X-ray film for 7 days at room temperature. Panel A: Cellular proteins. Lanes 1-4, control. Lanes 5-8, 100ng/ml hFSH plus 10^{-6}M testosterone. Lanes 1 and 5, $1x10^5$ cells/well. Lanes 2 and 6, $2x10^5$ cells/well. Lanes 3 and 7, $3x10^5$ cells/well. Lanes 4 and 8, $4x10^5$ cells/well. Panel B: Secreted proteins. Lanes 1-6, control. Lanes 7-12, 100ng/ml hFSH plus 10^{-6}M testosterone. Lanes 1 and 7, $5x10^4$ cells/well. Lanes 2 and 8, $1x10^5$ cells/well. Lanes 3 and 9, $1.5x10^5$ cells/well. Lanes 4 and 10, $2x10^5$ cells/well. Lanes 5 and 11, $3x10^5$ cells/well. Lanes 6 and 12, $4x10^5$ cells/well. Proteins of interest, and their approximate molecular weights, are marked on the right.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B
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1 2 3 4 5 6 7 8 9 10 11 12
O
200
116
66
44
36
24
~240
~140

3.4 Effects of Antioestrogens

3.4.1 Progesterone Production

Fig. 2.19 shows cell numbers after treatment of cells with 10^{-5}M ICI 164,384 and ICI 164,275. No difference in numbers of cells attached was observed between control and treated groups. Fig. 2.20 shows the effect of increasing concentrations of ICI 164,384 on progesterone production stimulated by 100ng/ml FSH alone (Fig.2.20A), 100ng/ml FSH plus 10^{-7}M E_2 (Fig. 2.20B) and 100ng/ml FSH plus 10^{-7}M DHT (Fig. 2.20C). Although ICI 164,384 effectively, dose-dependently and reversibly inhibited the stimulatory effect of E_2 on FSH-stimulated progesterone production (Fig. 2.20B), the level to which it inhibited progesterone production was well below the level produced in response to 100ng/ml FSH alone. Furthermore, ICI 164,384 also inhibited the effects of FSH alone (Fig. 2.20A) and FSH plus DHT (Fig. 2.20C). The supposedly inactive compound ICI 164,275 was found to have the same, although less marked, effects as ICI 164,384 (Fig. 2.21).

Fig. 2.22 shows that phenol red had no effect on progesterone production by granulosa cells over the dose range of 10-100μg/ml, either alone or in the presence of 100ng/ml FSH. Note that medium 199 contains 20μg/ml phenol red, and MEM contains 10μg/ml.

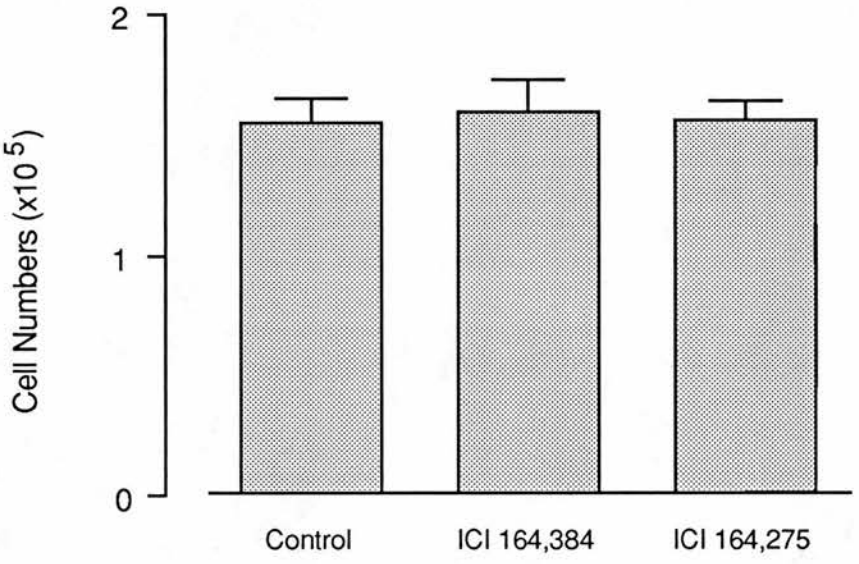


Fig.2.19. Effect of ICI 164,384 and ICI 164,275 (10^{-5}M) on numbers of rat granulosa cells attached to culture dishes after 48h of culture. Medium was removed, monolayers were washed once with 1ml MEM before being detached and counted. Values represent the mean (±SE) from counts from triplicate wells, counting 4 fields of a haemocytometer for each well. No statistically significant differences in cell numbers were observed between treatments.

Fig.2.20. Effect of increasing concentrations of ICI 164,384 on progesterone production by cultured rat granulosa cells, stimulated by 100ng/ml FSH alone (A), 100ng/ml FSH plus 10^{-7}M oestradiol-17β (B) or 100ng/ml FSH plus 10^{-7}M 5α-dihydrotestosterone (C). Shaded bars on the right (X) show progesterone production by cells cultured in the presence of equimolar (10^{-6}M) concentrations of ICI 164,384 and oestradiol-17β (B) or 5α-dihydrotestosterone (C), to demonstrate reversibility of effect of ICI 164,384. Values represent the mean (±SE) from twelve wells, from four separate experiments. In each graph, points sharing the same superscript are not significantly different from one another ($p<0.05$).

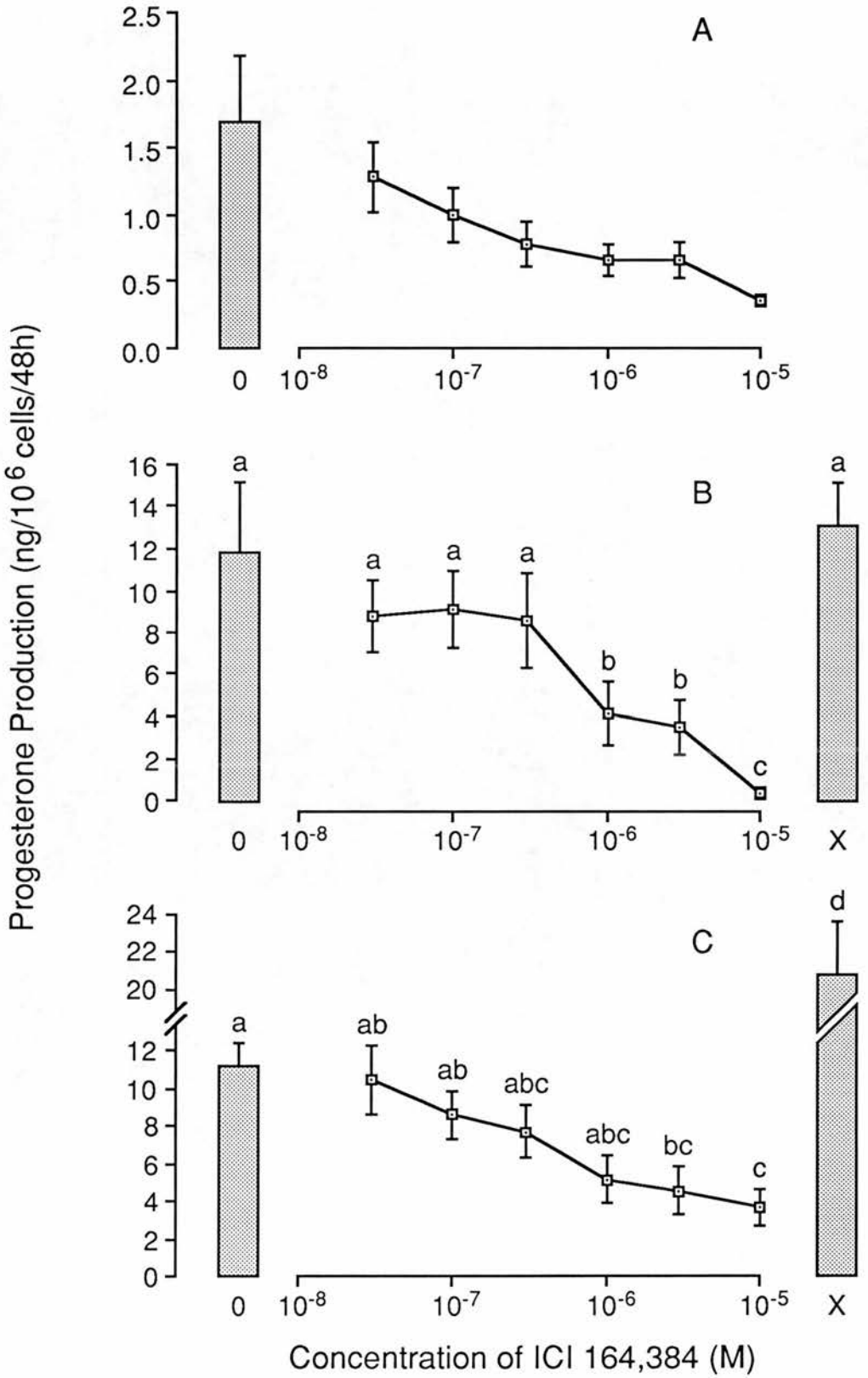
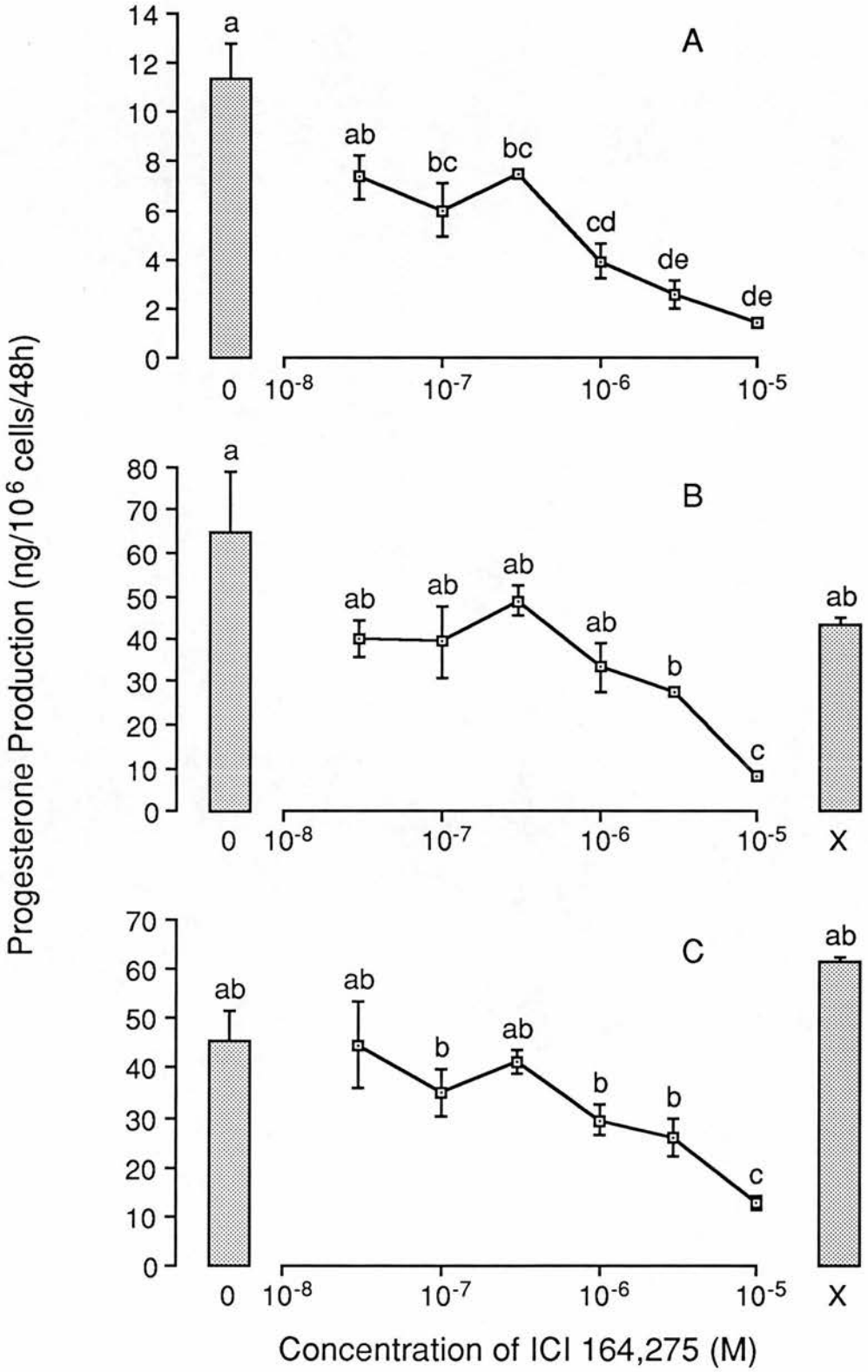

A
B
C
Progesterone Production (ng/10 6 cells/48h)
Concentration of ICI 164,384 (M)
0
10-8
10-7
10-6
10-5
X
a
a
a
a
b
b
c
a
ab
ab
abc
abc
bc
c
d

Fig.2.21. Effect of increasing concentrations of ICI 164,275 on progesterone production by cultured rat granulosa cells, stimulated by 100ng/ml FSH alone (A), 100ng/ml FSH plus 10^{-7}M oestradiol-17β (B) or 100ng/ml FSH plus 10^{-7}M 5α-dihydrotestosterone (C). Shaded bars on the right (X) show progesterone production by cells cultured in the presence of equimolar (10^{-6}M) concentrations of ICI 164,275 and oestradiol-17β (B) or 5α-dihydrotestosterone (C), to demonstrate reversibility of effect of ICI 164,275. Values represent the mean (±SE) from triplicate wells. In each graph, points sharing the same superscript are not significantly different from one another ($p<0.05$).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10^{-8}
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10^{-5}
X
Progesterone Production (ng/10^6 cells/48h)
Concentration of ICI 164,275 (M)

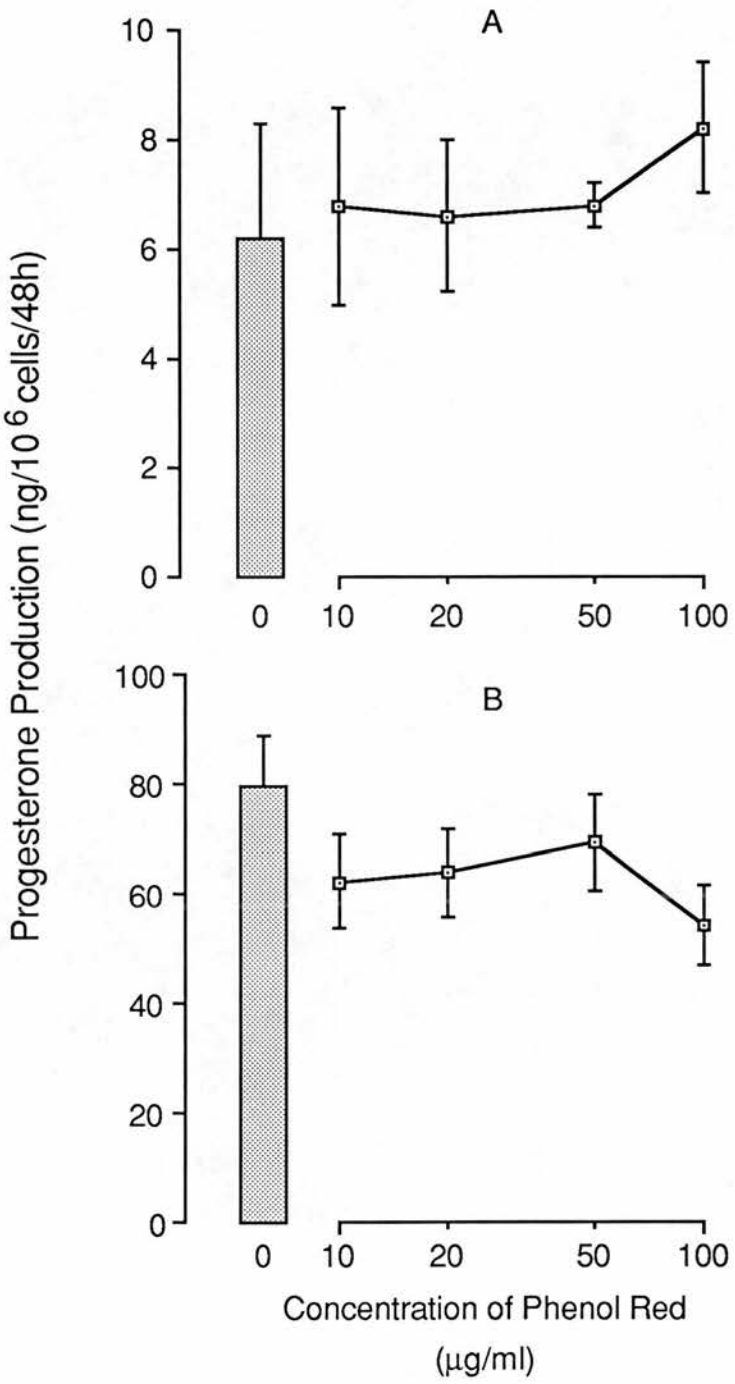


Fig.2.22. Effect of increasing concentrations of phenol red on progesterone production by cultured rat granulosa cells, stimulated by 100ng/ml FSH alone (A) or 100ng/ml FSH plus 10^{-7}M 5α-dihydrotestosterone (B). Values represent the mean (±SE) from triplicate wells. No significant effect of phenol red was observed.

3.5 Influence of DES Pretreatment

3.5.1 Progesterone Production

Fig. 2.23 shows *in vitro* progesterone production in response to FSH and steroids by granulosa cells isolated from control animals and animals which had been implanted *in vivo* with DES. Responsiveness of both groups of cells to FSH plus T was readily observed, although the magnitude of the response of the DES-pretreated cells was greater than that of the control cells.

3.5.2 Protein Synthesis

Fig. 2.24 shows an autoradiograph of an SDS-PAGE of cellular and secreted granulosa cell protein samples from control animals or animals pretreated for 4 days with DES. No difference in the pattern of protein secretion or the responses to FSH and FSH plus T were seen.

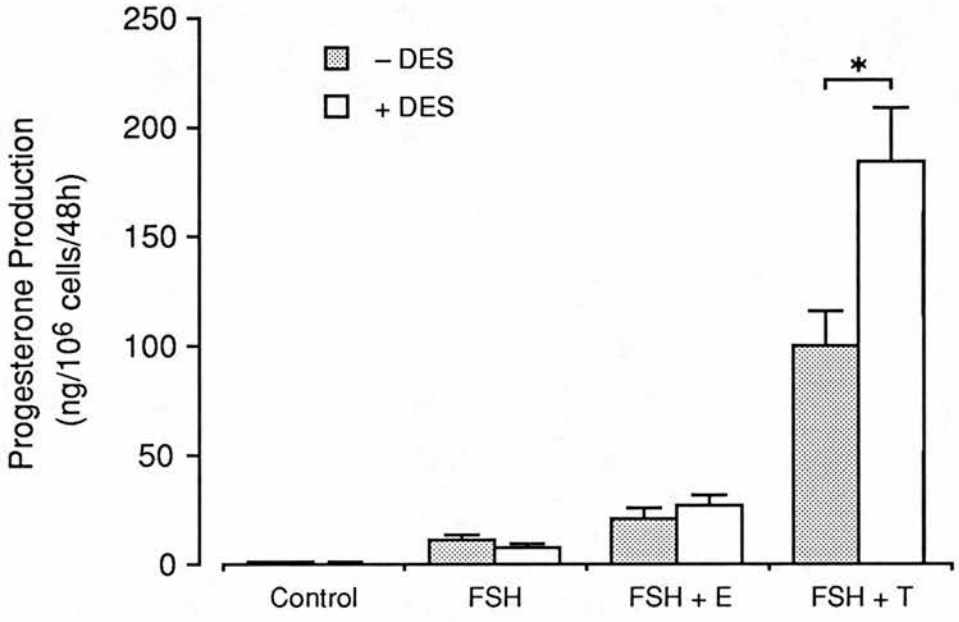
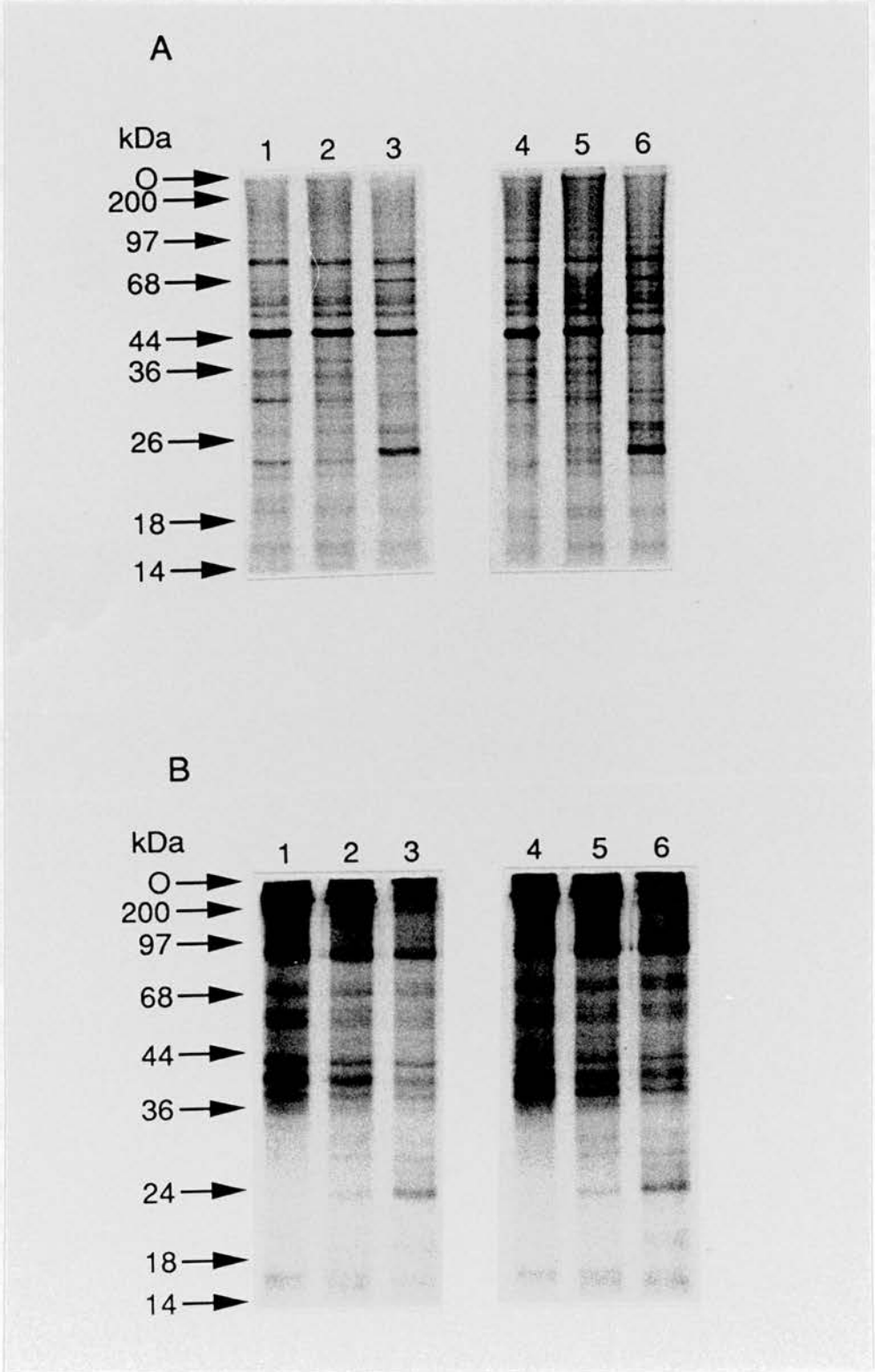


Fig.2.23. Effect of treatment of animals *in vivo* with DES on subsequent *in vitro* steroidogenic responsiveness of granulosa cells to FSH and steroids. Treated animals were treated with DES for 4 days before animals were killed and granulosa cells isolated. Cells from control (filled bars) and treated animals (open bars) were then cultured for 48h in the absence (Control) or presence of 100ng/ml hFSH alone (FSH), or in combination with 10^{-6}M oestradiol-17β (FSH + E_2) or testosterone (FSH + T). Values represent the mean (±SE) from nine wells from three separate experiments. Asterisks denote statistically significant differences in progesterone production between cells from control and DES-treated animals ($p<0.05$).

Fig.2.24. Effect of treatment of animals *in vivo* with DES on subsequent patterns of cellular (A) and secreted (B) protein synthesis *in vitro*. Cells from control animals (lanes 1-3) or DES-treated animals (lanes 4-6) were cultured for 48h in the absence (lanes 1 and 4) or presence of 100ng/ml hFSH (lanes 2 and 5) or 100ng/ml hFSH plus 10^{-6}M testosterone (lanes 3 and 6) before the labelling period. $2x10^4$ (A) or 10^4 cpm (B) were subjected to SDS-PAGE in 12.5% polyacrylamide gels. Cellular proteins were visualised by direct autoradiography for 5 days at room temperature. Secreted proteins were visualised by fluorography for 5 days at -70C.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24
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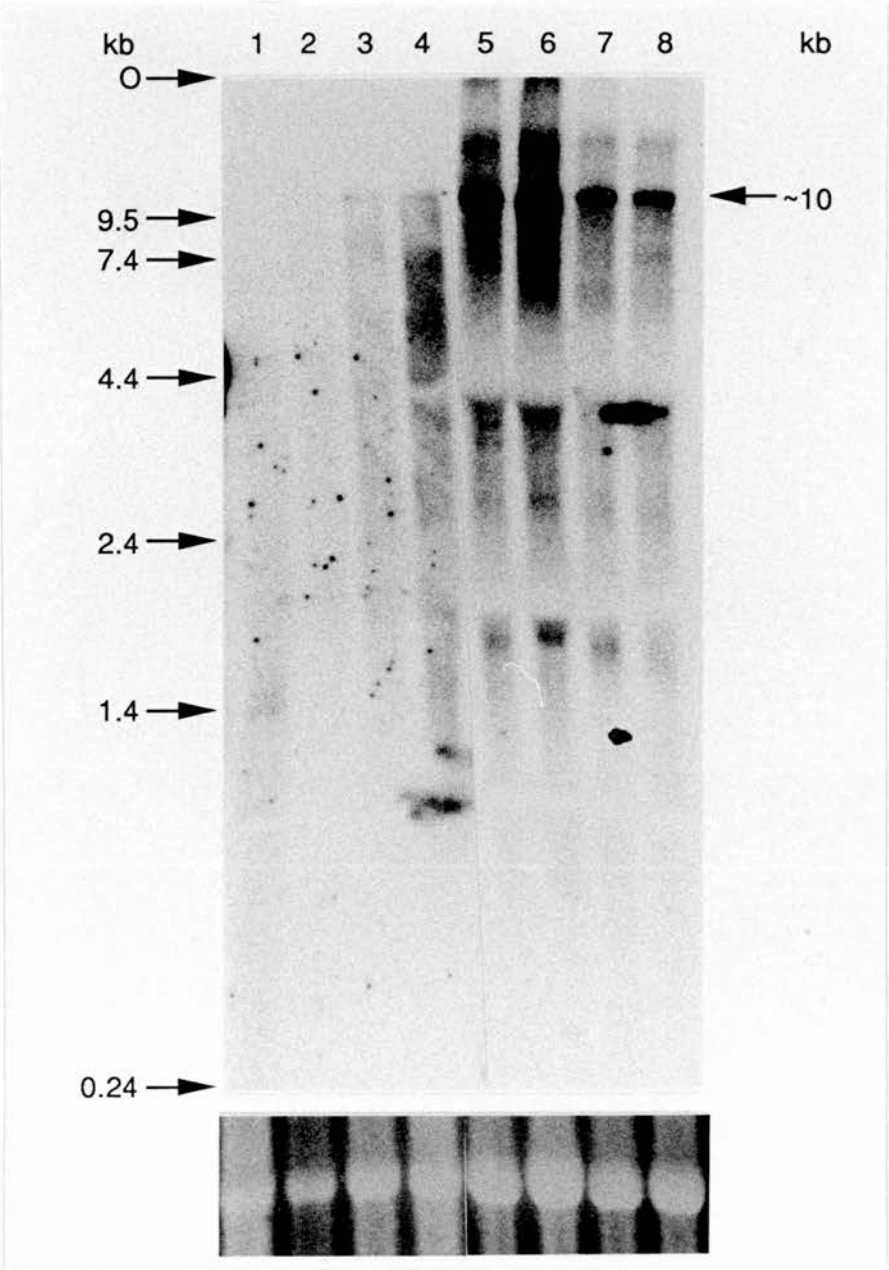
14

3.6 Expression of mRNA Encoding Steroid Receptors

Fig.2.25 shows a Northern blot which was probed using a cDNA probe to the rat androgen receptor. No hybridisation of this probe to spleen or uterus RNA was observed. One band of RNA with an approximate molecular weight of 10kb, in agreement with previously published reports (Tan *et al.* 1988; Lubahn *et al.* 1988; Namiki *et al.* 1991), was detected with the androgen receptor probe in prostate, adrenal and granulosa cell RNA. Levels of androgen receptor mRNA were highest in RNA extracted from granulosa cells. The expression of androgen receptor mRNA did not appear to be influenced by gonadotrophin treatment either *in vivo* or *in vitro*. Differences in intensity of hybridisation to granulosa cell RNA on this blot can be explained in terms of differences in amounts of RNA loaded in each lane.

Fig.2.26 shows a Northern blot probed with an RNA probe complementary to mRNA coding for the steroid-binding domain of the mouse oestrogen receptor. Full length oestrogen receptor mRNA (~6.5kb) was detected in mouse and rat uterus and ovary, although the signal was much less intense in ovary than uterus. However, a smaller RNA species (~1.5kb) present only in ovary RNA hybridised strongly to the probe. The full length oestrogen receptor mRNA was detected in rat ovary, and was somewhat enriched in residual ovary RNA samples, and was absent from granulosa cell RNA samples. The smaller transcript, however, was not detected in residual ovary RNA, and was greatly enriched in granulosa cell RNA. The abundance of this oestrogen receptor-related transcript was also found to be influenced by treatment of animals *in vivo* with gonadotrophins. Treatment of animals with hCG caused a slight increase in the abundance of this transcript, although this was not certain, since loading of total granulosa cell RNA from hCG-treated animals was slightly greater than that of the other samples. Treatment with FSH caused a greater increase in the level of this transcript. Treatment with hCG following treatment of animals with FSH led to a fall in levels of this molecule to or below control levels. Another even smaller RNA species present only in spleen RNA also hybridised to the probe, although

Fig.2.25. Expression of androgen receptor mRNA in rat tissues, and its regulation *in vivo* in granulosa cells. RNA was extracted from tissues as described in section 2.6.1, and 20μg of each sample were subjected to electrophoresis in a 1.5% agarose/formaldehyde gel, and transferred to a nylon filter. The blot was probed with a ^{32}P-labelled rat androgen receptor cDNA probe, washed at 65C, and exposed to x-ray film for 7 days. Lane 1, spleen; lane 2, uterus; lane 3, prostate; lane 4, adrenal; lane 5, granulosa cells from control animals; lane 6, granulosa cells from animals treated *in vivo* with 20IU hCG 12h before isolation of cells; lane 7, granulosa cells from animals treated *in vivo* with four injections of 20μg oFSH at intervals of 12h, the last injection being given 12h before isolation of cells; lane 8, granulosa cells from animals treated *in vivo* with four injections of 20μg oFSH and one of 20IU hCG 12h before isolation of cells. All animals, except those from which prostate tissue was obtained, were treated *in vivo* with DES for 4 days. Migration of RNA molecular weight markers is indicated on the left. The 18s ribosomal RNA bands stained with ethidium bromide, from the gel used to generate the Northern blot are shown at the bottom, to show loading of RNA.


kb
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2
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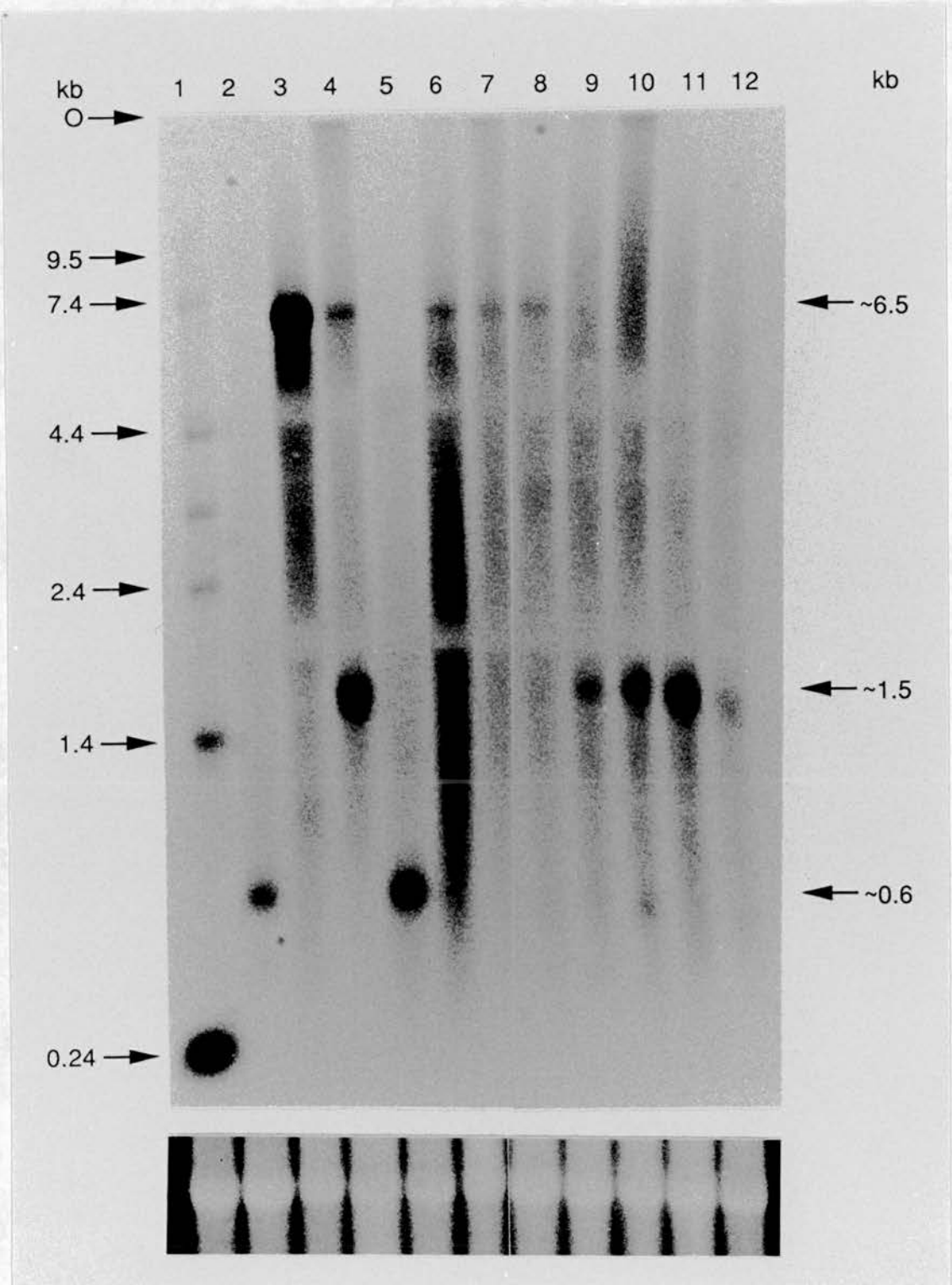
4
5
6
7
8
kb
O
9.5
7.4
4.4
2.4
1.4
0.24
~10

Fig.2.26. Expression of oestrogen receptor mRNA in rat and mouse tissues, and its regulation *in vivo* in rat granulosa cells. RNA was extracted from tissues as described in section 2.6.1, and 20μg were subjected to electrophoresis in a 1.5% agarose/formaldehyde gel, and transferred to a nylon filter. The blot was probed with a ^{32}P-labelled mouse oestrogen receptor cRNA probe, washed at 70C, and exposed to x-ray film for 5 days. Lane 1, molecular weight markers; lane 2, mouse spleen; lane 3, mouse uterus; lane 4, mouse ovary; lane 5, rat spleen; lane 6, rat uterus; lane 7, rat whole ovary; lane 8, rat ovary after isolation of granulosa cells; lane 9, granulosa cells from control rats; lane 10, granulosa cells from animals treated *in vivo* with 20IU hCG 12h before isolation of cells; lane 11, granulosa cells from animals treated *in vivo* with four injections of 20μg oFSH, the last injection being given 12h before isolation of cells; lane 12, granulosa cells from animals treated *in vivo* with four injections of 20μg oFSH and one of 20IU hCG 12h before isolation of cells. All rats were treated *in vivo* with DES for 4 days. Sizes of RNA molecular weight markers are indicated on the left. The 18s ribosomal RNA bands stained with ethidium bromide, from the gel used to generate the Northern blot are shown at the bottom, to show loading of RNA.


kb
1 2 3 4 5 6 7 8 9 10 11 12
kb
O
9.5
7.4
4.4
2.4
1.4
0.24
~6.5
~1.5
~0.6

hybridisation was not observed after washing of blots at 70C, whereas hybridisation to the ~1.5kb species was reduced but not lost at this temperature (not shown).

4 Discussion

These studies demonstrate that effects of FSH on granulosa cell function can be observed using cultured granulosa cells from rats pretreated *in vivo* with oestrogen. FSH administered *in vivo* led to increased production of progesterone, induced LH/CG responsiveness, and caused major changes in protein synthesis. However, exposure of granulosa cells *in vitro* to FSH alone stimulates progesterone production to only a modest degree and caused slight changes in patterns of protein synthesis, suggesting that the effects of FSH are facilitated, or even mediated, by other factors *in vivo*. Effects of FSH on both of these parameters of granulosa cell differentiation are markedly and dose-dependently augmented by simultaneous treatment of cells with sex steroids. In agreement with the literature (Hudson *et al.* 1987; Hillier & deZwart, 1981), the rank order of potency of the four steroids studied in stimulating progesterone production was E_2<2-OH-E_2<DHT<T. These results underline the extreme potency of sex steroids in augmenting the effect of FSH on these markers of *in vitro* differentiation of granulosa cells, in particular production of progesterone. Because the technique of SDS-PAGE is not quantitative, no conclusions about the relative potencies of these steroids in stimulating changes in patterns of protein synthesis could be reached.

The strongly stimulatory effect of the combination of FSH and testosterone was not due in any way to a stimulation of proliferation, since this combination did not cause any change in cell numbers. If cell monolayers are washed before recovery and counting of cells, it appears that stimulated cultures contain fewer cells than controls, implying that treatment of cells with FSH and testosterone leads to reduced cell proliferation, or increased cell death. Reduced proliferation is unlikely, since numbers observed in control cultures after 48h in culture are close to the numbers initially added. If cell monolayers are not washed before

recovery and counting of cells, however, cell numbers in control and stimulated groups are the same, indicating that many of the cells in the stimulated groups were not attached to the culture dishes. It could be argued that those unattached cells are dead, but there is good evidence that FSH treatment leads to aggregation and rounding of granulosa cells in culture (Amsterdam & Rotmensch, 1987), causing cells to detach from the substrate (see Chapter 3). Trypan blue exclusion could not be used to assess viability of cells in these experiments because almost all cells in both control and stimulated groups took up the dye after trypsinisation, possibly due to the enzymatic disruption of gap junctions between cells.

Timecourse experiments revealed that although effects of FSH and steroids on granulosa cell progesterone production (but not protein synthesis) could be detected after only 6h in culture, the greatest accumulation of progesterone occurred during the second day. Thereafter, the rate of progesterone production was seen to fall. This may be due to loss of responsiveness after prolonged exposure to hormones, or it may be that 72h is an excessive length of time to culture these cells without changing the medium.

Patterns of protein synthesis are also dependent on the length of exposure of cells to hormones prior to labelling. As on progesterone production, the effects of FSH and steroids were most apparent after 48h of treatment. The changes in patterns of protein secretion by control cells changed gradually with time in culture, the ~200kDa and the ~24kDa proteins being lost, and the ~240kDa protein being induced, by 48h preincubation. However, the pattern of cellular protein synthesis followed a different pattern. The patterns of cellular protein synthesis by freshly isolated cells and control cells incubated for 6, 48 and 72h prior to labelling were very similar, with the exception of the ~24kDa protein, which was synthesised less with longer incubation. The pattern of cellular protein synthesis after 24h of preincubation was strikingly different from these patterns, two proteins of ~120kDa and ~100kDa molecular weight being induced, and a less abundant ~70kDa protein being lost. These observations were reproducible, and are difficult to explain. Possibly they reflect an episode of mitotic activity, or a change in morphology in response to being placed in culture. Mitotic activity is

unlikely, since these cells do not appear to proliferate in this culture system, and since these cells continue to secrete progesterone in response to FSH and steroids at that time. The lack of effect of FSH on cellular protein synthesis at this time-point does suggest that the cells undergo some process at this time which overrides the normal response to FSH. Intriguingly, a clear effect of FSH and testosterone on secreted protein synthesis, and indeed on progesterone production, was apparent after 24h preincubation. This suggests that the process overriding the response to FSH and testosterone which affects cellular proteins does not prevent the effects of FSH on steroidogenesis or secretory protein patterns. On the basis of these results, it was decided that future experiments would be conducted for 48h.

Patterns of protein synthesis and production of progesterone *in vitro* were also found to be dependent upon the plating density of the cells in culture. The accumulation of progesterone in the medium in response to FSH plus testosterone increased with cell density, reaching a plateau of ~500ng/ml at $3x10^5$ cells/well. Since no exogenous cholesterol substrate such as LDL was added to the medium, this apparent loss of responsiveness could have been due to a shortage of substrate for steroidogenesis at high cell density. Progesterone accumulation in response to FSH plus oestradiol and FSH plus 2-OH-E_2 bore the same relationship to cell density, increasing with cell density up to a plateau at $150x10^5$ cells/well. However, the maximum level reached was only ~90ng/ml in response to FSH plus E_2, and ~300ng/ml in response to FSH plus 2-OH-E_2, showing that the reduced apparent responsiveness at high cell density was not due to exhaustion of substrate. The reason for this phenomenon is not clear. Although progesterone production on a per-cell basis decreases with cell density at high densities, the amount of progesterone produced by stimulated cells at all cell densities was vastly greater than that produced by untreated cells (<1ng/ml in all cases).

Patterns of protein synthesis were found to be only slightly affected by plating density, the synthesis of the FSH-responsive ~240kDa secreted protein, which increases with cell density in particular. Therefore, the effect of FSH on the secretion of this protein was most easily seen at high cell density. Effects of FSH on the synthesis of the ~24kDa, ~32kDa and

~100kDa cellular proteins could be observed at all cell densities, although the inhibition of the synthesis of the ~45kDa cellular protein was only clear at high cell density. These results would suggest that high cell densities (>$4x10^5$ cells/well) are best for the study of FSH-induced changes in protein synthesis. However, at such high cell density, the yield of radiolabelled secreted protein was low, for reasons that are not clear. As discussed above, culture of cells at high plating density also results in some quantitative reduction in their steroidogenic response to FSH and steroids. High cell density also imposes practical limitations on experiments, in that more cells are required for each treatment, reducing the number of treatments which can be used in an experiment. As a trade-off between FSH-responsiveness, radiolabelled protein yield and numbers of experimental treatments, the cell density which was chosen for use in subsequent experiments was $2x10^5$ cells/well.

The magnitude of the steroidogenic response of granulosa cells to FSH and steroids *in vitro* may be somewhat increased by treatment of animals *in vivo* with DES. However, the strong augmentation by sex steroids of the effects of FSH on progesterone production was observed in all granulosa cell cultures, whether or not the animals had been treated with DES. *In vitro* patterns of protein synthesis and changes in patterns in response to FSH and steroid were also unaffected by treatment of animals with DES, so it was concluded that the use of DES treatment to increase yields of granulosa cells is justified for studies of the steroidal control of FSH-induced *in vitro* differentiation.

Although patterns of protein synthesis were not absolutely consistent between experiments, several consistently FSH-responsive proteins were observed. The most prominent of these proteins was the major ~24kDa protein present in both cell monolayers and in culture media, the synthesis of which was stimulated by FSH. The effect of FSH on the synthesis of this protein was augmented by all four sex steroids studied. The fact that this protein was present in samples of cellular proteins and was also secreted into the culture medium suggests that it is a secretory protein which is stored in relatively large amounts. A protein which migrates at the same molecular weight was synthesised and secreted by freshly isolated cells, and its synthesis and secretion was

strongly stimulated by treatment *in vivo* with FSH. The abundance of this protein, and its marked FSH-responsiveness both *in vitro* and *in vivo* suggest that it has an important role in granulosa cell function.

The other highly reproducible effect of FSH on synthesis of secreted proteins *in vitro* was the inhibition of synthesis of the very abundant ~240kDa protein. However, this protein was not synthesised by freshly isolated cells, and did not appear in cultured granulosa cell samples until they have been incubated for at least 24h. Furthermore, the synthesis of this protein was also dependent on the plating density of the cells, being a major secreted protein only at cell densities of $>1x10^5$ cells/well. These data suggest that this protein is produced in response to the conditions of cell culture. However, its FSH-responsiveness does suggest that it is of some functional significance.

The most clear and consistent FSH-induced changes in *in vitro* synthesis of cellular proteins, other than the induction of the ~24kDa protein, were the inhibition of synthesis of the ~100kDa protein and the induction of the ~65kDa protein. It is not clear whether FSH administered *in vivo* induces the same changes, but both these proteins were observed in samples of cellular protein synthesised by freshly isolated cells. The synthesis of an extremely abundant ~45kDa cellular protein was also frequently observed to be inhibited by treatment with FSH, both *in vitro* and *in vivo*, suggesting that this may also be an important protein.

It was hoped that the effects of oestrogen could be shown to be specific using antioestrogens. This would allow the presence or absence of oestrogen receptors in granulosa cells to be inferred, since it is still not clear whether the effects of oestrogen on granulosa cell steroidogenesis are receptor mediated.

The results of studies using antioestrogens were inconclusive. ICI 164,384 and ICI 164,275 were shown to have no partial agonistic (oestrogenic) activity in the granulosa cell culture model. ICI 164,384 appeared to act as an effective antioestrogen, dose-dependently and reversibly suppressing the augmentation of FSH-stimulated progesterone production by oestradiol. The level to which FSH and oestradiol-stimulated progesterone production was inhibited by 10^{-5}M ICI 164,384 was far below that stimulated by FSH alone, suggesting that this

compound inhibits more than just the stimulation by the exogenous oestrogen. This was confirmed by the fact that ICI 164,384 inhibits progesterone production stimulated by FSH alone and by FSH plus the nonaromatisable androgen, DHT. This inhibition was not due to toxicity of the antioestrogen because cell numbers attached after 48h of treatment were unaffected by the presence of any of the compounds tested at up to 10^{-6}M. This does not rule out the possibility that ICI 164,384 and ICI 164,275 may be toxic at the highest dose used (10^{-5}M). Nor was the antioestrogen inhibiting an oestrogenic effect of phenol red present in the culture medium, since this was shown to have no oestrogenic activity in this system. ICI 164,275, which is reported in other systems to be inactive as an antioestrogen, also dose-dependently and reversibly inhibited progesterone stimulated by FSH alone, FSH plus oestradiol or FSH plus DHT.

These results using the "pure" antioestrogens could be interpreted as evidence that the effects of oestrogen are not specific. However, it could also be argued that these results show that these compounds have nonspecific inhibitory effects on granulosa cell function, and are therefore not suitable for studies of this kind. If these effects are nonspecific, it is curious that these compounds display the same inhibitory properties as other, completely unrelated, antioestrogens in the absence of oestrogen (Kessel & Hsueh, 1987; Knecht *et al.* 1985). The fact that addition of excess oestrogen can reverse the effect of the ICI compounds suggests a specific and competitive inhibition of oestrogen action. The inhibition by the ICI compounds of progesterone production stimulated by FSH plus DHT was less profound, and the reversal with excess DHT more dramatic, than the inhibition of the response to FSH plus oestradiol, and its reversal with oestradiol. These results argue in favour of the explanation that the antioestrogen was acting to block the effect of endogenous oestradiol, produced in response to FSH and steroids, which is the conclusion reached by Knecht *et al.* (1985) to explain the inhibitory effects of tamoxifen and keoxifene in the absence of oestradiol. However, in the absence of aromatisable androgen substrate, synthesis of oestradiol by cultured granulosa cells is negligible (Hillier & deZwart, 1982; Hillier & deZwart, 1981). The androgen used in these cultures was

5α-dihydrotestosterone, which is unable to act as a substrate for oestrogen synthesis, and even acts as a competitive aromatase inhibitor (Wickings *et al.* 1987). Steroidogenic enzymes can be inhibited directly by high concentrations of steroids (Gower & Cooke, 1983), so it is possible that these compounds, which are steroids, may be acting as nonspecific inhibitors of progesterone synthesis, perhaps with a direct effect on the P-450scc enzyme. ICI 164,384 does not appear to be a useful tool for the study of the specificity of oestrogen regulation of granulosa cell function, and its use was abandoned.

The results of the studies on androgen receptor mRNA expression were less ambiguous. It was clearly shown that granulosa cells express significant levels of androgen receptor mRNA, which do not appear to be strongly influenced by treatment of animals with gonadotrophins *in vivo*. The levels of androgen receptor mRNA detected in granulosa cells were low, but were substantially higher than those detected in prostate RNA. Assuming that levels of this mRNA are reflected in the production of the androgen receptor, this relatively high level of gene expression may explain, at least in part, the extreme potency of androgens in modulating the action of FSH. The apparent lack of effect of gonadotrophins on levels of androgen receptor mRNA is consistent with the finding that the androgen receptor gene contains an SP1 binding site in its promoter region, characteristic of a "housekeeping" promoter (Tilley *et al.* 1990).

Although very low levels of oestrogen receptor mRNA of the expected molecular weight (~6.5kb) were detected in rat and mouse ovarian RNA, the present study failed to detect this message in rat granulosa cell RNA. However, a smaller transcript (~1.5kb), which was not present in uterus RNA samples, was detected in mouse ovary and rat granulosa cell RNA using a probe complementary to the region of the oestrogen receptor mRNA encoding the oestrogen-binding domain of the receptor. The full length oestrogen receptor mRNA was detected in RNA extracted from residual ovarian tissue, but the shorter transcript was not. The same results were found in the mouse in a previous study (Hillier *et al.* 1989a). In that study, this smaller transcript was shown not to be detected using a probe complementary to the part of the oestrogen

receptor mRNA encoding the amino terminus and DNA binding domain of the receptor. The authors also showed that this species is not a truncated form of the oestrogen receptor mRNA, based on the lower thermal stability of hybridisation of the probe to this message than to the authentic oestrogen receptor mRNA. The identity of this oestrogen receptor-related transcript is still unknown. Since it was only detected using a probe complementary to the region of the oestrogen receptor mRNA encoding the oestrogen-binding domain of the receptor, it is possible that this message encodes a protein capable of binding oestrogen. Oestrogen-binding species distinct from the oestrogen receptor have been reported previously in rat granulosa cells (Kudolo *et al.* 1984b; Kudolo *et al.* 1984a). Also, Hillier *et al.* (1989a) point out that regions of the androgen-binding protein (ABP) and sex hormone-binding globulin (SHBG) mRNAs share significant nucleotide sequence homology with regions of the oestrogen-binding domain of the mouse oestrogen receptor, and that the rat ABP mRNA is ~1.6kb in length. Therefore, it is possible that the ~1.5kb oestrogen receptor-related transcript is also related to ABP and SHBG. In contrast to the expression of androgen receptor mRNA, expression of oestrogen receptor-related RNA was found to be under the control of gonadotrophins. Administration of FSH or hCG *in vivo* led to an increase in the abundance of this RNA, and administration of hCG after prior FSH treatment caused a decrease in its abundance. This pattern of expression suggests that expression of the oestrogen receptor-related RNA increases with the maturity of the granulosa cells, and would predict that its expression would be greatest in the granulosa cells of preovulatory follicles, and would decrease after the LH surge. Since preovulatory follicles contain the highest levels of oestrogen, these findings are consistent with the speculative explanation for the existence of a cytoplasmic oestrogen-binding protein offered by Kudolo *et al.* (Kudolo *et al.* 1984a), who suggested that such a molecule may act to regulate intracellular oestrogen levels. However, the physiological significance of this oestrogen receptor-related transcript awaits its further characterisation.

In conclusion, the results of these experiments indicate that the DES-primed immature rat granulosa cell culture model is highly suitable for

studies on the role of paracrine factors such as sex steroids in the regulation of granulosa cell differentiation. Granulosa cells can be cultured at high cell density without greatly compromising their hormonal responsiveness, to yield sufficient material for studies of protein synthesis. Several differentiation-related changes in protein synthesis were observed, in particular increased synthesis of a ~24kDa protein which was secreted and stored in high abundance, increased synthesis of a ~65kDa cellular protein, reduced synthesis of a high molecular weight secreted protein and reduced synthesis of ~100kDa, ~45kDa and ~32kDa cellular proteins. The significance of these changes requires the identification of these proteins. The abundance of some of these proteins suggests that they may be structural proteins, and their FSH-responsiveness may reflect morphological changes occuring during the growth of the follicle. Based on a growing literature on the morphological aspects of granulosa cell differentiation, these observations were extended to studies on the regulation by FSH and steroids of synthesis of cytoskeletal and extracellular matrix proteins, which are described in the following chapter.

Chapter 3 Morphological Correlates of Differentiation *In Vitro*

1 Introduction

The experiments described in the previous chapter showed, using progesterone production as a marker of differentiated function, that granulosa cells maintained in culture can be induced to differentiate in response to FSH, and that steroids are potent enhancers of FSH action. It was also shown that this differentiation of granulosa cells *in vitro* is accompanied by changes in their gross patterns of protein synthesis. The synthesis of a number of abundant proteins was observed to be particularly strongly influenced by FSH treatment, and so the object of further study was to identify some of these proteins. Since cultured granulosa cells undergo dramatic changes in morphology during gonadotrophin stimulation, it was likely that some of these proteins were involved in this morphological differentiation.

1.1 Cytoskeleton, Extracellular Matrix and Cell Shape

Cell shape is determined by the cytoskeleton, which in turn is influenced by the extracellular matrix. The cytoskeleton is composed of three types of protein structure, microfilaments, microtubules and intermediate filaments. Microtubules are composed of tubulins (α and β) and microtubule-associated proteins (MAPs), and are primarily responsible for intracellular transport (e.g. of chromosomes during cell division, and of organelles) (Darnell *et al.* 1986). Intermediate filaments, whose constituents (such as vimentin, desmoplakin and various cytokeratins) vary between cell types, are probably involved in the control of cell shape, although disruption of intermediate filaments has been shown not to affect cell shape, motility or division (Birchmeier, 1984).

Microfilaments are certainly important in the determination of cell shape. Most cells grown in culture on plastic dishes will adopt a flattened shape (Freshney, 1987; Gospodarowicz *et al.* 1978), as a consequence of the formation of focal adhesion plaques between the cells and extracellular matrix (Watt, 1986; Hay, 1981). These plaques are formed by the binding of extracellular matrix proteins by cell surface receptors, which are coupled to microfilaments via the membrane-associated actin-binding protein vinculin (Otto, 1990; Darnell *et al.* 1986). The microfilaments are composed of polymerised actin filaments, with other proteins such as myosin, α-actinin and tropomyosins bound at intervals to the fibres, which act to stabilise and cross-link actin filaments into bundles known as stress fibres (Craig & Pollard, 1982). These stress fibres run between the adhesion plaques throughout the cytoplasm, and are responsible for the flattening of the cell. Cells can be induced to become rounded in culture by the use of agents such as cytochalasin B, which depolymerises actin microfilaments (Amsterdam & Rotmensch, 1987), microinjection of antibodies to actin (Birchmeier, 1984), or by transfection with the avian retrovirus, Rous Sarcoma Virus (RSV) (Kellie, 1988). The transformation by RSV is brought about by a protein kinase $pp60^{v\text{-}src}$, which phosphorylates several cytoskeletal proteins on tyrosine, especially vinculin, disrupting the connection of stress fibres to the membrane at the adhesion plaque (Kellie *et al.* 1986). In fact, vinculin has been shown to be a substrate for phosphorylation by protein kinases A and C, suggesting that modification of vinculin may be a common feature of hormonal stimulation of cells (Werth & Pastan, 1984; Schliwa *et al.* 1984; Turner *et al.* 1989).

In addition to its functions in the control of cell shape and motility, the cytoskeleton is involved in a variety of other functions. Many membrane proteins are associated with microfilaments, and so receptor mobility and turnover **are** affected by microfilaments. Mobility of membrane receptors in the membrane is necessary for their interaction with signal transducing proteins such as G-proteins and adenylate cyclase, so the cytoskeleton is very important in the control of hormone responsiveness and desensitisation (for review, see Zor, 1983). The

cytoskeleton is also involved in the regulation of water and ion balance within the cell and within cellular compartments (Cameron *et al.* 1988).

The cytoskeleton itself is influenced by the substrate to which the cell is attached. Cells form contacts with the proteins of the extracellular matrix by way of cell surface receptors, which recognise specific components of the extracellular matrix (Kellie, 1988; Watt, 1986). The exact composition of the extracellular matrix varies according to the cell type in question, but the major constituents are collagens, proteoglycans and fibronectin (Watt, 1986; Hay, 1981). The latter has been studied in some detail, and is used as an attachment factor in many cell culture systems (Freshney, 1987). Fibronectin is a glycoprotein which is present in serum, where it is involved in wound healing, and is also secreted by many cell types into the extracellular matrix (Yamada & Olden, 1978). It exists as a dimer or oligomer of similar subunits (Vartio & Vaheri, 1983). There are three types of subunit, all of which are derived from the same gene by alternative splicing, with molecular weights between 220 and 250kDa (Ruoslahti *et al.* 1982). *In vitro*, fibronectin promotes cell spreading and proliferation, and cells often take on a flattened morphology when cultured on fibronectin-coated plates (Yamada & Olden, 1978; Watt, 1986).

Cell shape has a profound influence on cell function and proliferation (Gospodarowicz *et al.* 1978). For example, macrophages, which are normally rounded, respond to prostaglandins with an increase in intracellular cAMP levels. However, when placed in culture, these cells will become flattened. Such cells no longer respond to prostaglandins, although there is no change in prostaglandin binding (Zor, 1983).

1.2 Morphological Differentiation of Granulosa Cells

It is well known that granulosa cells cultured on plastic adopt a flattened morphology in the absence of hormones, but become rounded and form multilayered aggregates upon treatment with FSH (Tsang *et al.* 1988; Ben Ze'ev *et al.* 1987; Morley *et al.* 1987; Amsterdam *et al.* 1981). In addition, microvilli form on the surface of the cells (Ben Ze'ev &

Amsterdam, 1986; Ben Ze'ev *et al.* 1987), gap junctions form between cells (Lindner *et al.* 1977; Amsterdam & Rotmensch, 1987), and cytoplasmic projections connect neighbouring aggregates of cells (Amsterdam & Rotmensch, 1987; Amsterdam *et al.* 1989). At the same time, FSH induces the expression of LH receptors, which are associated with these microvilli (Zor, 1983), and are most abundant on cells which have been induced to aggregate (Amsterdam *et al.* 1981). Lipid droplets accumulate in the cytoplasm, and smooth endoplasmic reticulum and large numbers of mitochondria develop around the nuclear region (Ben Ze'ev *et al.* 1987; Amsterdam *et al.* 1981; Soto *et al.* 1986). Steroid secretion increases as this cellular reorganization proceeds (Ben Ze'ev & Amsterdam, 1986). Studies using agents which disrupt cytoskeletal structures have further emphasised the involvement of the cytoskeleton in granulosa cell function. Steroid secretion by cultured granulosa cells is increased following disruption of microfilaments with antibodies to actin or using cytochalasin B (Tsang *et al.* 1988; Carnegie & Tsang, 1988), or disruption of microtubules with **colchicine** (Carnegie & Tsang, 1987; Carnegie *et al.* 1988). Steroid secretion by adrenal cells is also increased by **colchicine** (Temple & Wolff, 1973). However, the response of granulosa cells to gonadotrophins is reduced by disruption of microfilaments with cytochalasin B (Zor, 1983; Zor *et al.* 1978) or microtubules with **colchicine** (Carnegie *et al.* 1987; Zor, 1983; Zor *et al.* 1978). Interestingly, taxol, which stabilises microtubules (in contrast to the depolymerising effect of **colchicine**) has the same inhibitory effect as colchicene on gonadotrophin responsiveness in granulosa cells (Carnegie *et al.* 1987). Possibly these divergent effects of drugs which interfere with the cytoskeleton on steroid secretion and hormonal responsiveness are due on one hand to the role of the cytoskeleton in steroid transport and secretion, and on the other hand, to its role in receptor mobility and recycling (Zor *et al.* 1979; Zor, 1983). Because of the importance of the cytoskeleton in cell function, and the marked changes in morphology of granulosa cells which accompany their differentiation *in vitro*, a number of studies have concentrated on the regulation of several cytoskeletal and extracellular matrix proteins in granulosa cells.

It has been shown that fibronectin secretion by cultured granulosa cells is low at the start of culture, but increases with time in culture, such that after 72h, fibronectin becomes a major protein product of these cells (Skinner & Dorrington, 1984; Dorrington & Skinner, 1986). FSH treatment of cultured cells dramatically reduces fibronectin synthesis (Skinner *et al.* 1985; Carnegie, 1990) by a cAMP-dependent mechanism (Lobb & Dorrington, 1987; Dorrington & Skinner, 1986). A cAMP-responsive element (CRE) is situated in the 5' regulatory region of the bovine fibronectin gene, although cAMP analogues inhibit fibronectin mRNA expression and synthesis (Bernath *et al.* 1990). This effect requires other regulatory sequences, and also requires synthesis of an unidentified presumptive regulatory protein (Bernath *et al.* 1990). The influence of steroids on the effect of FSH on fibronectin synthesis has not been well studied, although one report showed that testosterone and oestradiol-17β had no effect alone, and it was stated (although the data were not shown) that neither steroid modified this effect of FSH (Skinner *et al.* 1985).

The changes in the synthesis of several microfilament proteins during granulosa cell differentiation have also been studied. Immunofluorescence studies have demonstrated that untreated cultured granulosa cells contain a well developed network of actin stress fibres throughout the cytoplasm, attached to vinculin-containing adhesion plaques (Ben Ze'ev & Amsterdam, 1986; Ben Ze'ev & Amsterdam, 1989). Following stimulation of these cells with FSH or cAMP analogues, the pattern of actin distribution became diffuse, and vinculin was no longer detected. With the loss of these stress fibres, the cells became rounded. The synthesis of actin, α-actinin, vinculin and tropomyosins fell, and was associated with a fall in the levels of translatable mRNA encoding all these proteins (Ben Ze'ev *et al.* 1987; Ben Ze'ev & Amsterdam, 1987; Ben Ze'ev *et al.* 1989). In addition, it has been shown by timecourse experiments that the drop in synthesis of these proteins correlates with acquisition of steroidogenic responsiveness (Ben Ze'ev & Amsterdam, 1986; Amsterdam *et al.* 1989; Skinner *et al.* 1985).

The effects of steroids in modulating the effect of FSH on cytoskeletal protein expression have not been studied, although one study has shown that androgens prevent flattening of granulosa cells in

culture (Anderson *et al.* 1988). Therefore, the aim of the work described in this chapter was to identify some of the cytoskeletal and extracellular matrix proteins involved in the morphological changes accompanying granulosa cell differentiation, and to examine the effects of sex steroids on the control by FSH of these proteins.

2 Materials and Methods

Animals, hormones, cell culture method, extraction and analysis of RNA, and electrophoresis of protein samples were as described in chapter 2. Except for the timecourse experiment described below, all culture experiments were conducted for 48h, with a 24h metabolic labelling incubation in immunoprecipitation experiments. The following additional methods were employed in the experiments described in this chapter.

2.1 Immunoprecipitation

Vinculin was immunoprecipitated from cultured granulosa cell lysates, and fibronectin was immunoprecipitated from culture media, by a method based on those of Anderson & Blobel (1983), Kessler (1981) and McSween & Eastwood (1981). A monoclonal mouse IgG1 antibody to smooth muscle vinculin from chicken gizzard (clone no. VIN-11-5, Sigma), and a polyclonal goat antiserum raised against rat fibronectin (Calbiochem, La Jolla, CA) were used at dilutions of 1:50 and 1:20, respectively. Equal amounts of radioactivity (10-100µl) from each sample were mixed with SDS to give a final concentration of 0.5% (w/v) and boiled for 1min. Two volumes of buffer containing 1.25% (v/v) Triton X-100, 0.19M NaCl, 5mM EDTA, 25mM Tris/HCl pH 7.4 and 10KIU/ml aprotinin was added. Non-specific protein A binding in the samples was preabsorbed for 60min with 15µl of protein A (10% crude *Staphylococcus* cell suspension [Sigma] in buffer as above) and 10µl of normal mouse (cell lysates) or goat (media) serum. Samples were centrifuged and supernatants transferred to fresh tubes. Antibody was added to each sample to the desired dilution, and tubes were incubated overnight at 4C.

To act as a non-immune control, one tube in each series received a volume of normal mouse or goat serum (Scottish Antibody Production Unit [SAPU]) equal to the volume of antiserum added to the other tubes. Protein A was "prearmed" with rabbit antiserum to goat IgG (Sigma) or sheep antiserum to mouse IgG (SAPU) by mixing two volumes of protein A suspension with one volume of antiserum, and incubating for 2h at 4C. This mixture was centrifuged and the supernatant dicarded. The pellet was then resuspended to the original volume of protein A suspension in "wash" buffer (0.1% [v/v] Triton X-100, 0.02% [w/v] SDS, 0.19M NaCl, 25mM Tris/HCl pH 7.4, 5mM EDTA, 10KIU/ml aprotinin). 50µl of this pre-armed protein A suspension was then added to each sample, and incubated at room temperature for 4h, with occasional shaking. Samples were then centrifuged at 5,000g for 2min and supernatants discarded. The pellets were then resuspended in 1ml "wash" buffer. This procedure was repeated at least four times with "wash" buffer as above, and then once in detergent-free "wash" buffer, in order to remove nonspecific protein A binding. Final pellets were resuspended in 70µl of dissociating sample buffer (see chapter 2) and boiled for 5min to dissociate the protein of interest from the protein A. Tubes were centrifuged at 13,000g for 2min and supernatants (containing immunoprecipitated antigen) were transferred to fresh tubes. 30µl of each sample was subjected to SDS-PAGE as described in chapter 2, section 2.5.3, and immunoprecipitated protein was detected by autoradiography of dried gels, as described in chapter 2, section 2.5.3.

2.2 Northern Analysis of Actin mRNA

Mouse β-actin cDNA, cloned in pSP64 plasmid, was donated by Dr. M.G. Parker, ICRF, Lincoln's Inn Fields, London. The ~2kb cDNA insert was liberated with *Pst*I, gel purified and labelled for use as a probe by random priming as described in chapter 2, sections 2.6.3 and 2.6.4. Prehybridisation and hybridisation were carried out at 55C and washing was carried out at 65C (see chapter 2, section 2.6.6).

3 Results

The effect of FSH on the morphology of cultured granulosa cells is shown in Fig.3.1. Control (Fig.3.1A) and testosterone treated (Fig.3.1B) cells formed a monolayer of flattened cells. FSH (100ng/ml) caused some rounding and aggregation (Fig.3.1C), an effect which was enhanced by simultaneous treatment with 10^{-6}M testosterone (Fig.3.1D). Cells treated with both FSH and testosterone formed large aggregates of rounded cells several cells thick, with cytoplasmic projections extending between cells of neighbouring aggregates.

Immunoprecipitation of vinculin (Fig.3.2) yielded one faint band migrating at an approximate molecular weight of 130kDa, which was not precipitated in the absence of vinculin antiserum. A prominent ~45kDa protein was present in all precipitates, including the non-immune precipitate. Treatment of cells with 10^{-6}M testosterone alone had no effect on the abundance of this protein. Treatment with 100ng/ml FSH caused a decrease in the synthesis of vinculin, and simultaneous treatment with 10^{-6}M testosterone augmented the action of FSH. The amount of nonspecific precipitation of the ~45kDa protein also followed the same pattern.

The abundant ~240kDa secreted protein was identified by immunoprecipitation as fibronectin (Fig.3.3). The immunoprecipitation of fibronectin was carried out by Dr. P.T.K. Saunders, whom I thank for its use in Fig.3.3. This protein was not precipitated in the absence of specific fibronectin antiserum. Treatment of cells with 10^{-6}M testosterone alone had no effect on the abundance of this protein. Treatment with 100ng/ml FSH caused a decrease in the synthesis of fibronectin, and simultaneous treatment with 10^{-6}M testosterone augmented the action of FSH.

Attempts to identify actin by this method were unsuccessful. Therefore, the expression of this protein was studied by measurement of the steady-state levels of its mRNA. Fig.3.4 shows a Northern blot bearing total cellular RNA from cultured granulosa cells, probed with a ^{32}P-labelled mouse β-actin cDNA probe. Two RNA species were detected with this probe; one highly abundant band approximately 2.2kb in length, assumed to be β-actin mRNA, and one much weaker ~1.65kb band,

Fig.3.1. Phase contrast photomicrographs of granulosa cells, isolated from immature DES-treated rats, after 48h in culture. Treatments were as follows: A) control, B) 10^{-6}M testosterone alone, C) 100ng/ml FSH and D) 100ng/ml FSH plus 10^{-6}M testosterone. Magnification: x150.

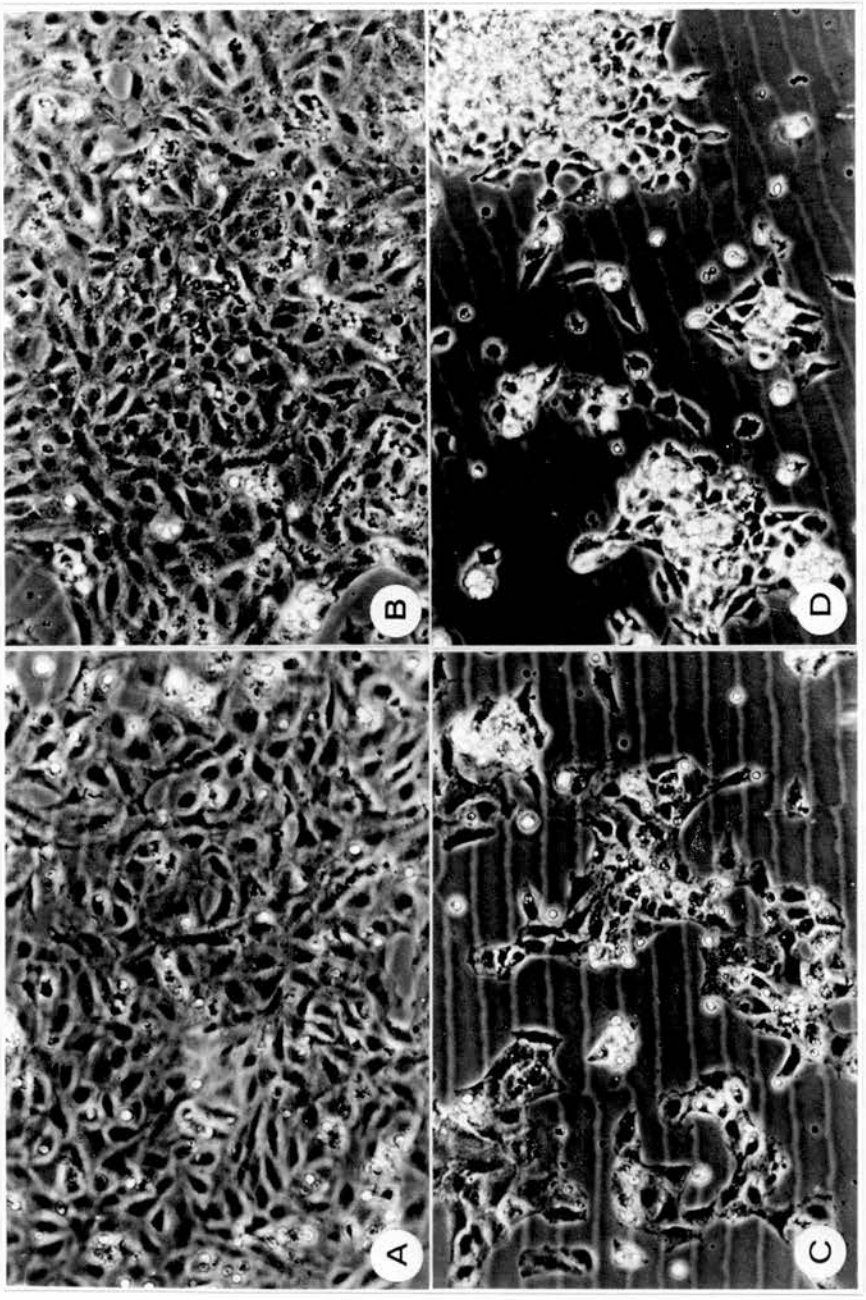

B
D
A
C

Fig.3.2. Immunoprecipitation of vinculin from samples of metabolically labelled rat granulosa cell proteins. Cells were cultured for 48h in the presence or absence of hormones, and then for a further 24h in the presence of ^{35}S-labelled methionine, in addition to hormonal treatments. Cells were lysed, and vinculin was immunoprecipitated from the lysates using a specific antiserum. Precipitated protein was then subjected to electrophoresis and autoradiography. Lane 1) total cellular protein from untreated cells; lane 2), non-immune precipitate, in which normal serum was substituted for specific antiserum; lanes 3-6), protein immunoprecipitated from cell lysates using vinculin antiserum. Treatments were as follows: lane 3) control, lane 4) 10^{-6}M testosterone, lane 5) 100ng/ml FSH, and lane 6) 100ng/ml FSH plus 10^{-6}M testosterone. Position of origin (O), and migration of molecular weight (in kDa) standards are marked on the left.

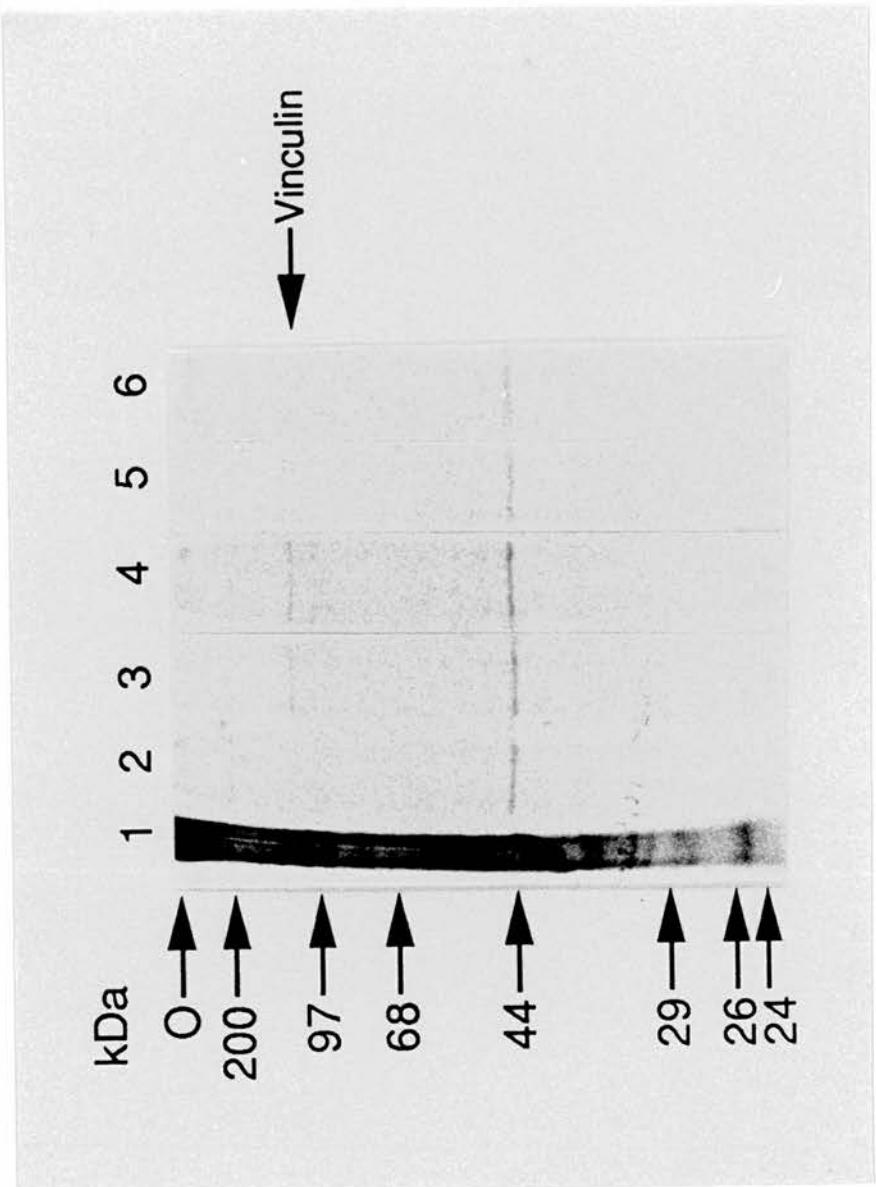

Vinculin
1 2 3 4 5 6
kDa
0
200
97
68
44
29
26
24

Fig.3.3. Immunoprecipitation of fibronectin from samples of metabolically labelled rat granulosa cell proteins. Cells were cultured for 48h in the presence or absence of hormones, and then for a further 24h in the presence of ^{35}S-labelled methionine, in addition to hormonal treatments. Medium was removed, and fibronectin was immunoprecipitated from the medium using a specific antiserum. Precipitated protein was then subjected to electrophoresis and autoradiography. Lane 1) total secreted protein from untreated cells; lane 2), non-immune precipitate, in which normal serum was substituted for specific antiserum; lanes 3-6), protein immunoprecipitated from culture medium using fibronectin antiserum. Treatments were as follows: lane 3) control, lane 4) 10^{-6}M testosterone, lane 5) 100ng/ml FSH, and lane 6) 100ng/ml FSH plus 10^{-6}M testosterone. Position of origin (O), and migration of molecular weight (in kDa) standards are marked on the left.

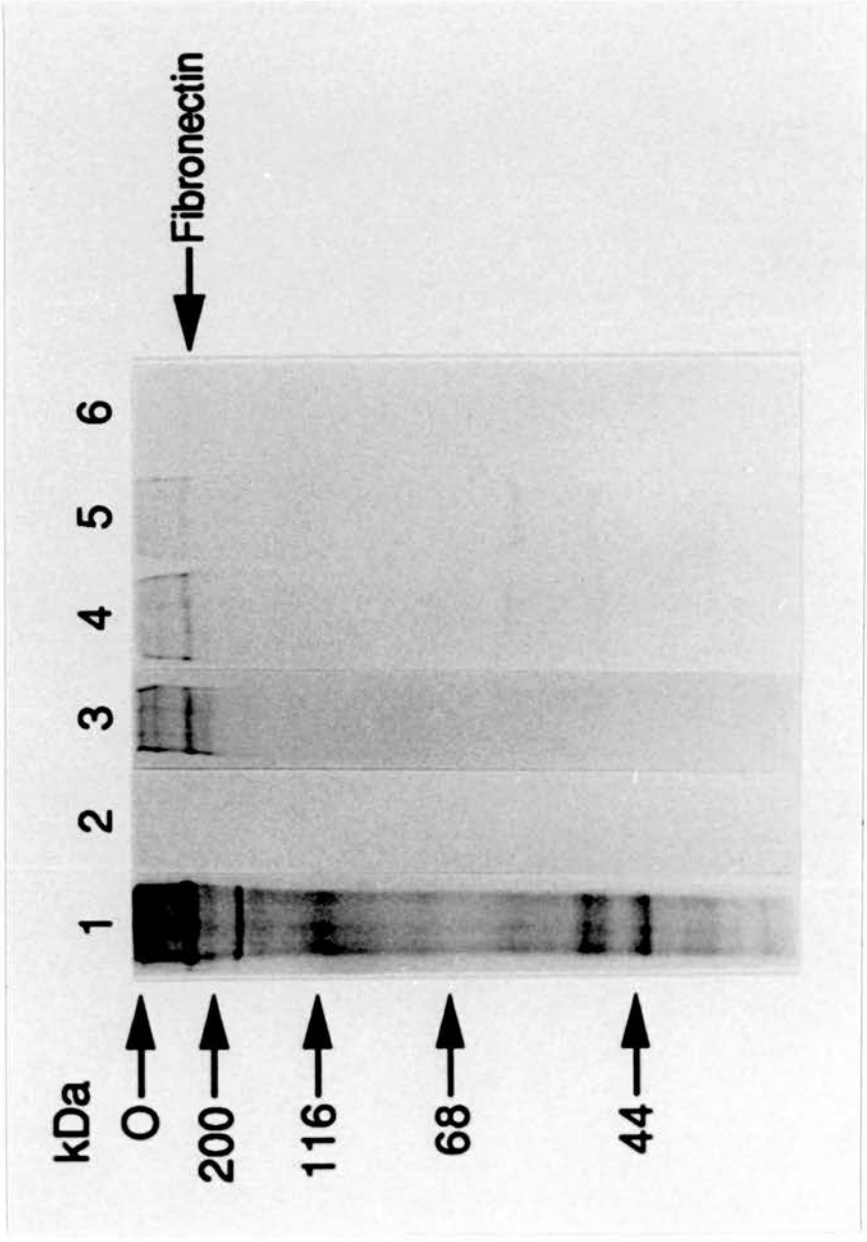
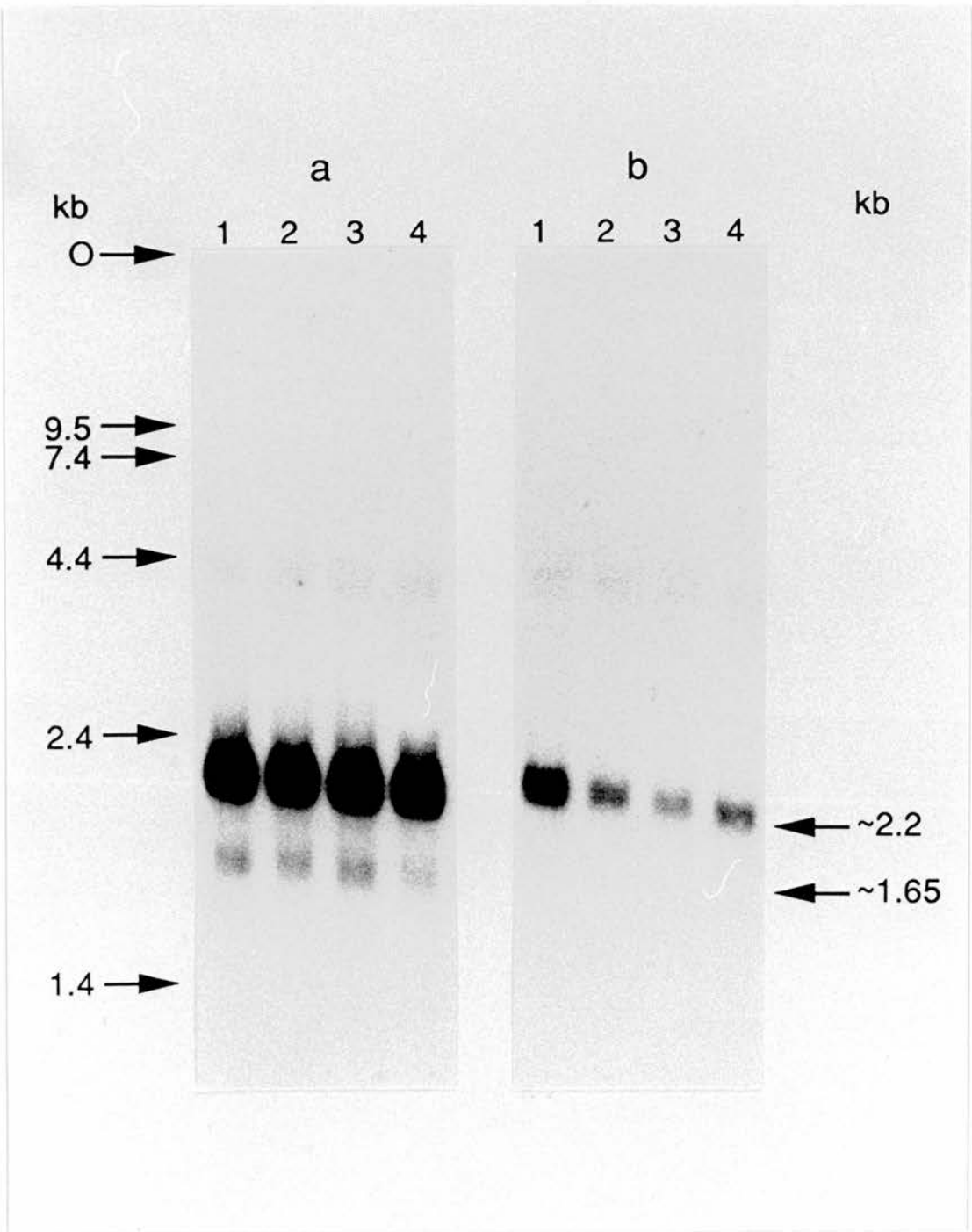

Fibronectin
6
5
4
3
2
1
kDa
0
200
116
68
44

Fig.3.4. Effects of FSH and steroids on granulosa cell actin mRNA levels *in vitro*. Granulosa cells were cultured for 48h in the presence or absence of hormones. Culture medium was removed, cells were lysed with solution D, and RNA was isolated from lysates by phenol:chloroform extraction. Four micrograms of total cellular RNA were subjected to electrophoresis in a 1.5% agarose gel containing 6.6% formaldehyde. RNA was transferred to a nylon membrane, and hybridised to a ^{32}P-labelled mouse β-actin cDNA probe at 55C overnight. The blot was washed at 65C twice for 30min, and exposed to x-ray film for 24h with one intensifying screen. Cells were treated as follows: panel a) no FSH, and panel b) 100ng/ml FSH, lane 1) no steroid, lane 2) 10^{-6}M testosterone, lane 3) 10^{-6}M 5α-dihydrotestosterone, and lane 4) 10^{-6}M oestradiol-17β. Migration of RNA molecular weight (in kb) standards is marked on the left.


a
b
kb
1 2 3 4
1 2 3 4
kb
O
9.5
7.4
4.4
2.4
1.4
~2.2
~1.65

assumed to be γ-actin mRNA, since α-actins are present only in muscle cells. Treatment of cells *in vitro* with oestradiol-17β, testosterone or 5α-dihydrotestosterone alone (10^{-6}M) had no effect on the level of actin mRNA. Treatment with 100ng/ml FSH alone caused a moderate decrease in β-actin mRNA. All three steroids tested strongly enhanced the inhibition of actin mRNA expression caused by FSH. Fig.3.5 shows that FSH had little effect on levels of β-actin mRNA at a concentration of 30ng/ml, and that the augmentative effect of androgen was dose-dependent, being apparent only at a steroid concentration of 10^{-6}M. Fig.3.6 shows that the *in vitro* effects of FSH and steroids on actin mRNA levels were unaffected by prior treatment of animals with DES. The timecourse of this effect of FSH was investigated by allowing cells to become flattened in culture in the absence of hormones, treating cells with 100ng/ml FSH, and extracting RNA from cells 1, 2, 4, 8, 24, and 48h after treatment. It was found that the effect of FSH, although modest in this experiment was apparent within 2h of treatment (Fig.3.7). Thereafter, levels of actin mRNA did not change.

The relationship of these findings with the pattern of actin mRNA levels *in vivo* was investigated. Fig.3.8 shows a Northern blot bearing total RNA extracted from cells freshly isolated from animals which had been treated *in vivo* with FSH and/or hCG. It was found that neither gonadotrophin administered *in vivo* had any effect on levels of actin mRNA.

Fig.3.5. Dose-dependent augmentation by DHT of the effect of FSH on granulosa cell actin mRNA levels *in vitro*. Granulosa cells were cultured for 48h in the presence or absence of hormones. Culture medium was removed, cells were lysed with solution D, and RNA was isolated from lysates by phenol:chloroform extraction. Ten micrograms of total cellular RNA were subjected to electrophoresis in a 1.5% agarose gel containing 6.6% formaldehyde. RNA was transferred to a nylon membrane, and hybridised to a ^{32}P-labelled mouse β-actin cDNA probe at 55C overnight. The blot was washed at 65C twice for 30min, and exposed to x-ray film for 24h with one intensifying screen. Cells were treated as follows: panel a) no FSH, and panel b) 30ng/ml FSH, lane 1) no steroid, lane 2) 10^{-8}M 5α-dihydrotestosterone, lane 3) 10^{-6}M 5α-dihydrotestosterone. Migration of RNA molecular weight (in kb) standards is marked on the left.

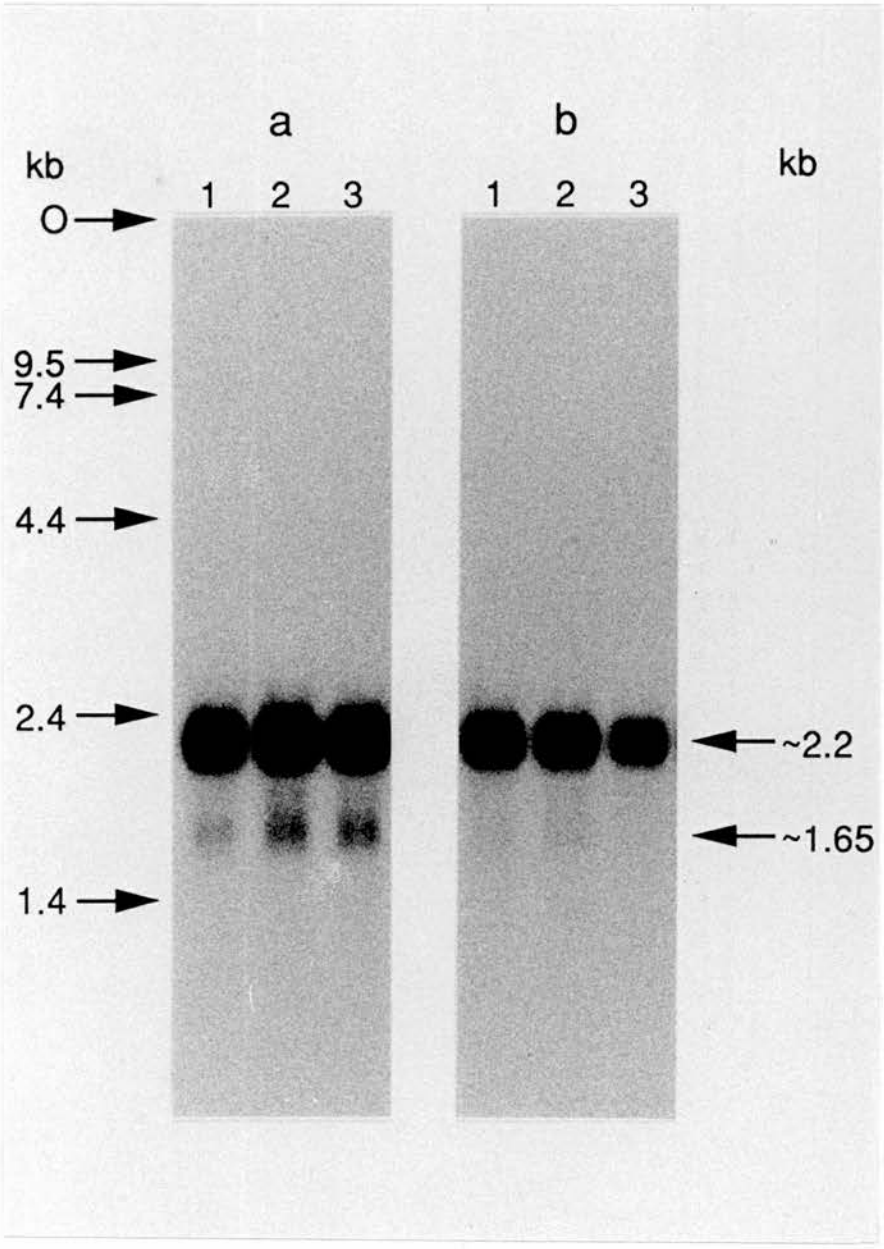

a
b
kb
1 2 3
1 2 3
kb
O
9.5
7.4
4.4
2.4
1.4
~2.2
~1.65

Fig.3.6. Effect of pretreatment of animals with DES on the regulation of granulosa cell actin mRNA levels by FSH and steroids *in vitro*. Granulosa cells from control animals and DES-treated rats were cultured for 48h in the presence or absence of hormones. Culture medium was removed, cells were lysed with solution D, and RNA was isolated from lysates by phenol:chloroform extraction. Five micrograms of total cellular RNA were subjected to electrophoresis in a 1.5% agarose gel containing 6.6% formaldehyde. RNA was transferred to a nylon membrane, and hybridised to a ^{32}P-labelled mouse β-actin cDNA probe at 55C overnight. The blot was washed at 65C twice for 30min, and exposed to x-ray film for 24h with one intensifying screen. Animals were either not treated (panel a), or treated (panel b) for 4 days with DES *in vivo*. Cells were treated as follows: lane 1) control, lane 2) 10^{-6}M testosterone, lane 3) 10^{-6}M oestradiol-17β, lane 4) 30ng/ml FSH, lane 5) 30ng/ml FSH plus 10^{-6}M testosterone, lane 6) 30ng/ml FSH plus 10^{-6}M oestradiol-17β. Migration of RNA molecular weight (in kb) standards is marked on the left.

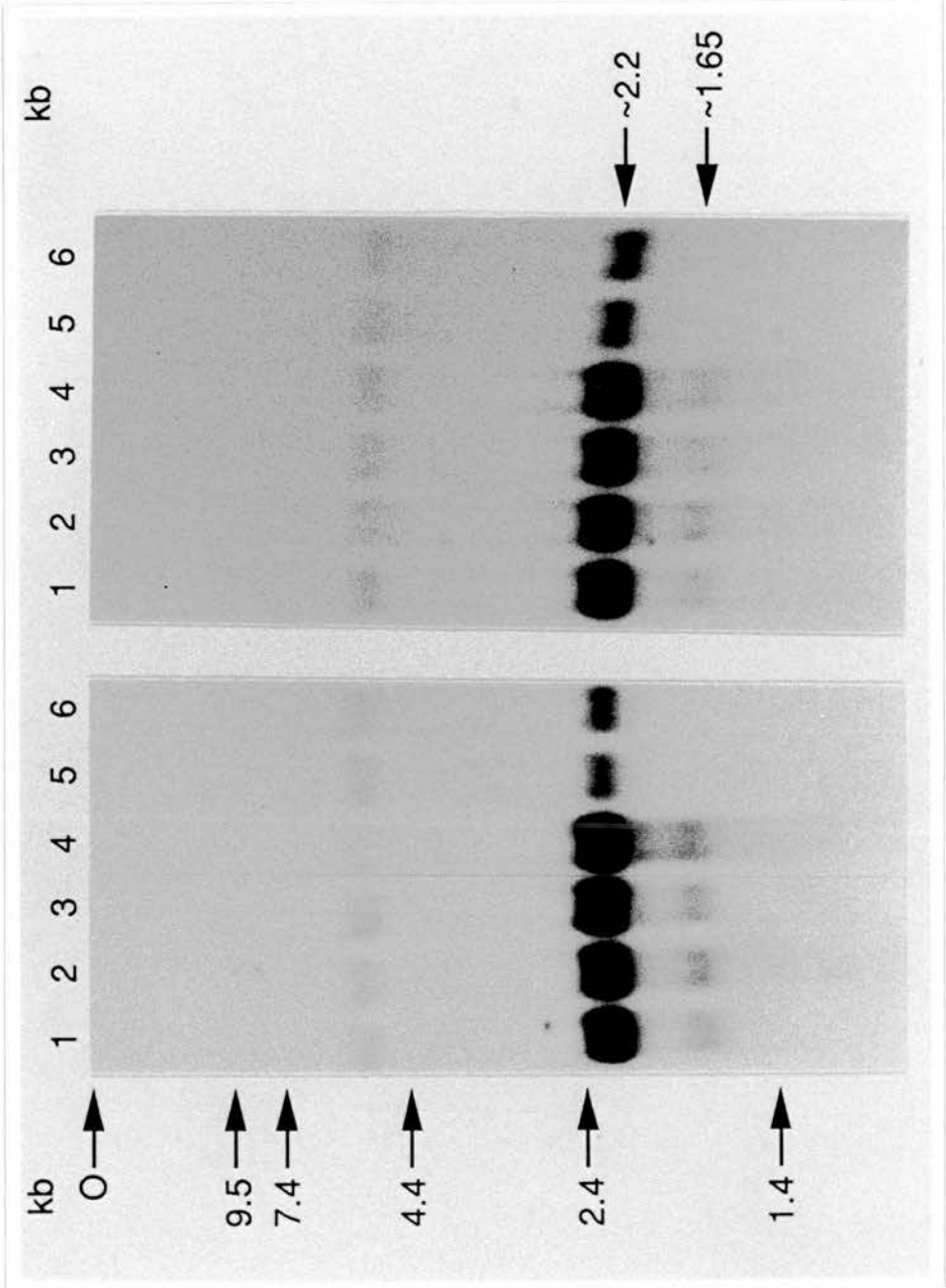

kb
~2.2
~1.65
1 2 3 4 5 6
1 2 3 4 5 6
kb
O
9.5
7.4
4.4
2.4
1.4

Fig.3.7. Time-course of the suppression of granulosa cell actin mRNA levels by FSH *in vitro*. Cells were allowed to attach overnight in the absence of hormones, and medium was replaced with medium containing 30ng/ml FSH. Culture medium was removed before stimulation (lane 1), or 1h (lane 2), 2h (lane 3), 4h (lane 4), 8h (lane 5), 24h (lane 6) or 48h (lane 7) after stimulation, cells were lysed with solution D, and RNA was isolated from lysates by phenol:chloroform extraction. Five micrograms of total cellular RNA were subjected to electrophoresis in a 1.5% agarose gel containing 6.6% formaldehyde. RNA was transferred to a nylon membrane, and hybridised to a ^{32}P-labelled mouse β-actin cDNA probe at 55C overnight. The blot was washed at 65C twice for 30min, and exposed to x-ray film for 24h with one intensifying screen. Migration of RNA molecular weight (in kb) standards is marked on the left.

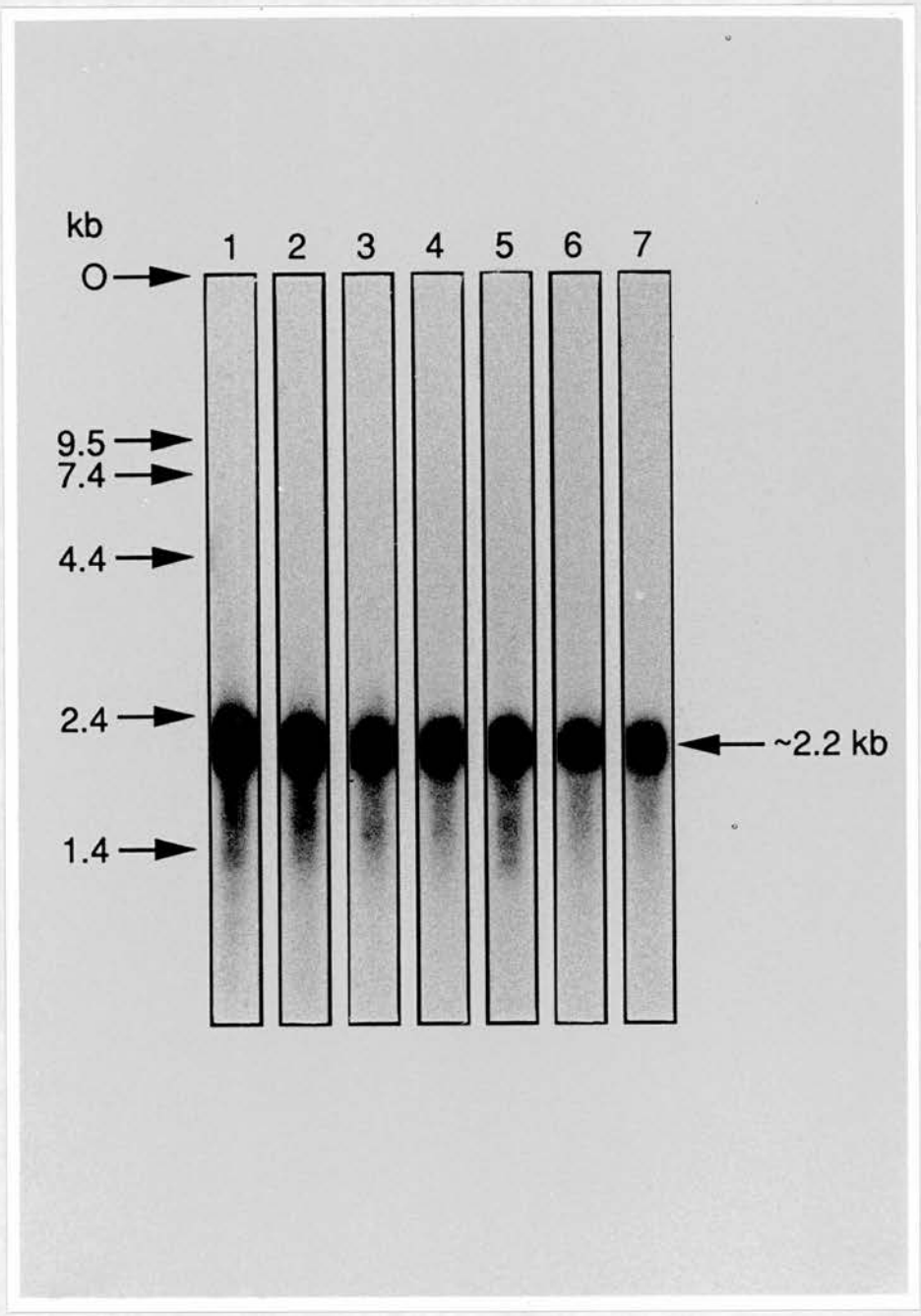
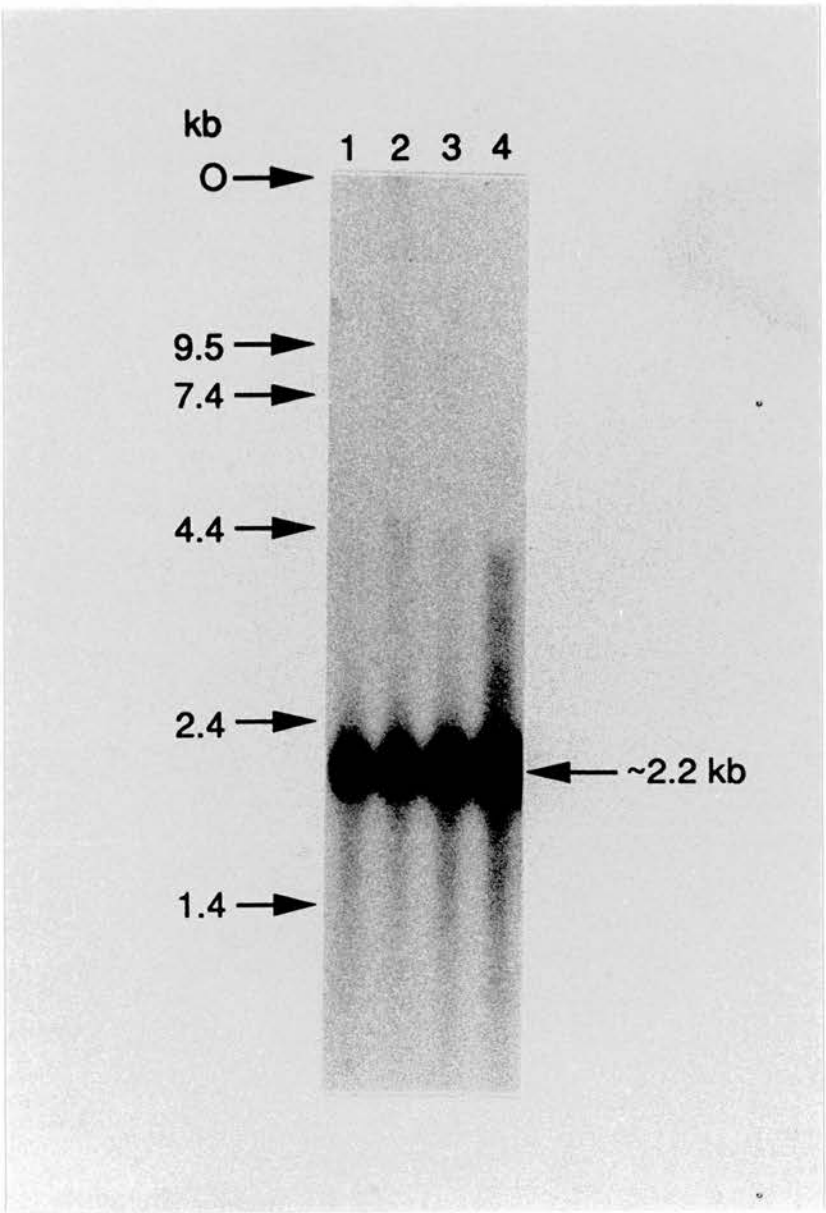

kb
1 2 3 4 5 6 7
O
9.5
7.4
4.4
2.4
1.4
~2.2 kb

Fig.3.8. Effects of gonadotrophins on granulosa cell actin mRNA levels *in vivo*. Immature female rats were treated with DES for four days and gonadotrophins as follows: lane 1) control, lane 2) one injection of 20IU hCG 12h before isolation of cells, lane 3) 20μg oFSH every 12h for the 48h prior to cell isolation, and lane 4) 20μg oFSH every 12h for 48h, plus one injection of 20IU hCG 12h before isolation of cells. Freshly isolated granulosa cells were lysed with solution D, and RNA was isolated from lysates by phenol:chloroform extraction. Five micrograms of total cellular RNA were subjected to electrophoresis in a 1.5% agarose gel containing 6.6% formaldehyde. RNA was transferred to a nylon membrane, and hybridised to a ^{32}P-labelled mouse β-actin cDNA probe at 55C overnight. The blot was washed at 65C twice for 30min, and exposed to x-ray film for 48h with two intensifying screens. Migration of RNA molecular weight (in kb) standards is marked on the left.


kb
1 2 3 4
O
9.5
7.4
4.4
2.4
1.4
~2.2 kb

4 Discussion

The results presented in this chapter have confirmed that FSH causes marked changes in morphology of cultured granulosa cells, and that these changes are associated with a decrease in fibronectin and vinculin synthesis and actin gene expression, as published studies have shown previously. Furthermore, they have also shown that androgens and oestrogens are capable of enhancing the effects of FSH on these markers of morphological differentiation. However, in contrast to the findings of Anderson *et al.* (1988), testosterone alone (10^{-6}M) had no effect on the morphology of cultured granulosa cells. The effects of FSH and steroids on actin mRNA levels were most closely studied, and it was found that FSH dose-dependently reduces the levels of actin mRNA in cultured granulosa cells, and that the modulatory effect of androgen, at least, is also dose-dependent. Pretreatment of animals *in vivo* with DES did not affect the subsequent response of granulosa cells to steroids *in vitro* with regard to actin mRNA levels. The effect of FSH on actin gene expression is rapid, occuring within 2h, although not profound. Immunofluorescence and electrophoretic studies of actin protein expression in granulosa cells have suggested that the changes in cell shape which occur in response to FSH are not accompanied by a complete loss of actin, but rather a redistribution (Ben Ze'ev & Amsterdam, 1986; Ben Ze'ev & Amsterdam, 1989). The effect of FSH and steroids on synthesis of fibronectin and vinculin appears to be more profound, synthesis of these proteins being barely detectable after treatment of cells with FSH and testosterone. This may be because vinculin and fibronectin are both required only for cell attachment, whereas the function of actin is less specialised, being the major component of microfilaments, which are required for functions other than cell attachment.

During immunoprecipitation of vinculin, a protein of approximately 45kDa was non-specifically precipitated. This protein was the most abundant protein in granulosa cell lysates. Being non-specific, one would expect the amount of this protein precipitated to remain unchanged between samples. However, the amount precipitated followed

the same pattern as vinculin. This may suggest that the efficiency of immunoprecipitation varied between samples, invalidating the conclusions drawn from the experiment. However, by comparison with published work on the electrophoresis of granulosa cell proteins, which demonstrated that actin, which is a 45kDa protein in its monomeric form, is the most abundant of granulosa cell proteins, it is likely that this non-specifically precipitated protein is actin. This was not confirmed, since attempts to immunoprecipitate actin failed, due to a failure to remove the ~45kDa protein from non-immune precipitates. Actin is a protein which interacts with a very large number of other proteins (Craig & Pollard, 1982), so it is likely that this is the non-specifically precipitated protein. If this is so, then the variation in the amount of this protein precipitated would be expected to follow the same pattern as that of vinculin, as was observed.

The regulation of actin gene expression in granulosa cells *in vivo* is quite different from that *in vitro*. In fact, no effect of gonadotrophins, alone or in combination, on actin mRNA levels was observed. Localisation of actin in sections of follicles has shown that there is very little actin in the granulosa or theca interna layers of the follicle, although the theca externa, which is a far more fibrous layer, is rich in actin (Lindner *et al.* 1977). In contrast to the *in vitro* data discussed in this chapter which suggests that actin synthesis by granulosa cells falls with increasing maturity, the only *in situ* granulosa cells in which actin has been detected are those nearest to the basement membrane within large follicles (Cran & Musk, 1985; Familiari *et al.* 1987), which might be expected to be the most highly differentiated cells in the follicle. *In vitro* studies suggest that fibronectin production by granulosa cells also falls with increasing maturity, but this, too, is not the case *in vivo*. Granulosa cell fibronectin production has been shown to be greatest in cells from large follicles (Carnegie, 1990; Reinthaller *et al.* 1990). Fibronectin synthesis has not been demonstrated in freshly isolated immature granulosa cells (Skinner & Dorrington, 1984; Skinner *et al.* 1985) (see also Fig.2.14), and is heavily dependent upon the plating density of granulosa cells *in vitro* (see Fig.2.19). These discrepancies suggest that the changes in expression of cytoskeletal and extracellular matrix proteins seen during *in*

vitro differentiation of granulosa cells may be artefactual. Certainly, the morphology of granulosa cells cultured in the absence of hormones does not resemble that of granulosa cells present in ovarian follicles. However, after stimulation of cultured granulosa cells with FSH, their morphology becomes closer to their morphology *in vivo*; that is, they become rounded and form aggregates several cell layers thick. It seems probable that granulosa cells will flatten in culture in the absence of hormones since no specialised functions are operating other than normal metabolic activity, but they must take on their *in vivo* morphology in order to manifest their differentiated functions, such as steroidogenesis. The flattened morphology of unstimulated granulosa cells in culture could therefore be seen as an artefact of placing these cells in culture, which must be overcome during stimulation by FSH in order that they may differentiate functionally. This interpretation is supported by the relatively rapid drop in actin mRNA, compared to the slower acquisition of maximal steroidogenic activity (Ben Ze'ev & Amsterdam, 1986; Skinner *et al.* 1985) (see also Fig.2.12).

The composition of the extracellular matrix is very variable between tissues, and cells from a particular tissue can be made to retain their normal *in vivo* morphology *in vitro* if they are cultured on an extracellular matrix derived from the same tissue (Watt, 1986). Therefore, this problem might be overcome by creating conditions in culture which more closely resemble the environment of granulosa cells within the follicle. Culture of granulosa cells on plates coated with fibronectin, which promotes cell spreading, lessens the effect of FSH on both morphology and steroidogenesis of these cells (Morley *et al.* 1987). However, if cells are cultured in collagen gels (Ben Rafael *et al.* 1988; Carnegie *et al.* 1988), or on extracellular matrix (ECM) derived from corneal epithelium (Furman *et al.* 1986; Ben Ze'ev & Amsterdam, 1986; Amsterdam *et al.* 1989), their round morphology is retained even in the absence of FSH. Basal production of progesterone by these cells is higher than that of cells cultured on plastic or fibronectin-coated plastic.

To date the detailed composition of the follicular basement membrane has not been analysed, and may prove difficult to reproduce routinely in culture dishes, and so the *in vitro* approach remains to some

extent an approximation to the true environment of the follicle. Despite the discrepancy between the production of cytoskeletal and extracellular matrix proteins by granulosa cells *in vivo* and their production *in vitro*, suggesting that granulosa cells may not behave *in vitro* as they do *in vivo*, these cells retain their responsiveness to gonadotrophins in culture. Therefore, although cultured granulosa cells may be different morphologically from those within a follicle, there is no evidence that the mechanisms controlling their function are qualitatively different. It was concluded that the regulation of cytoskeletal protein expression probably does not reflect physiological phenomena during follicular development. Therefore, these studies were discontinued in favour of studies of the regulation of the expression of functionally important granulosa cells proteins, of which the most interesting and potentially important were deemed to be the inhibins and activins.

A technical point which was evident from the studies described above concerns the widespread use of actin mRNA as an internal control in Northern blotting experiments. Although the technique of Northern blotting is not a quantitative one, some proof that equal amounts of RNA are present on each lane of a membrane is required. To demonstrate equal loading of gels and even transfer of RNA to Northern blots, the membranes are often probed for actin mRNA. Indeed, in some cases, autoradiographic signals are measured by densitometry, and the level of the mRNA of interest in each sample is expressed as a ratio of the intensity of the actin signal in the same lane. However, at least in cultured rat granulosa cells, actin mRNA is a quite inappropriate internal control to use, since its levels change so dramatically during differentiation.

Chapter 4 Inhibin Gene Expression

1 Introduction

In the previous chapter, FSH and steroids were shown to have marked effects on the morphology of granulosa cells, which are associated with profound changes in the expression of cytoskeletal and extracellular matrix proteins. However, evidence was found that these changes may not reflect physiological changes occurring in the follicle *in vivo*, but are probably artefacts of the *in vitro* approach. Therefore, little further understanding of ovarian physiology can be gained by pursuing such studies. However, it was shown in chapter 2, and in numerous published reports that many parameters of granulosa cell function, such as production of steroids and acquisition of LH responsiveness brought about by FSH, are retained by these cells when maintained in culture. With the caveat that cultured granulosa cells may not behave exactly as they do *in vivo*, it is clear that this model is useful for detailed study of the regulation of granulosa cell function (rather than structure) by FSH and steroids.

One of the most interesting aspects of granulosa cell function to emerge in recent years is their ability to produce inhibin. Current knowledge of the biology of inhibin is reviewed in detail in Chapter 1, Section 6.2.4. There are several reasons why inhibin biology is interesting. Firstly, like oestradiol, inhibin is capable of acting as a classic endocrine hormone, by suppressing secretion of FSH by the pituitary (Ying, 1988). As such inhibin is the only non-steroidal endocrine hormone known to be secreted by the ovary. According to current understanding, as discussed in chapter 1, suppression of FSH secretion by products of the developing follicle is central to the selection of dominant follicles (Baird, 1987), so inhibin may act in concert with oestradiol in the endocrine control of follicle selection. Secondly, like androgen and oestrogen, inhibin has also been shown to have direct paracrine effects within the ovary; in

particular, it has been shown to stimulate thecal androgen synthesis (Hsueh *et al.* 1987; Hillier *et al.* 1991b). The third major reason why inhibin is interesting is the fact that the β-subunits of inhibin are capable of forming β-β dimers, known as activins, which have opposite effects to inhibin in all systems studied to date (Ying, 1988). Furthermore, activins are closely related to other factors which have been shown to have direct effects on ovarian cells, and on a diverse range of differentiative processes in a variety of tissues and species. Intuitively, one would expect such ubiquitous molecules to be of importance in the regulation of the function of tissues in which they are expressed. Unlike inhibin, and like oestradiol, activins have stimulatory effects on granulosa cells. In immature cells, activin augments the action of FSH on several markers of differentiation (Hutchinson *et al.* 1987; Sugino *et al.* 1988a; LaPolt *et al.* 1989; Xiao *et al.* 1990; Xiao & Findlay, 1991). This effect suggests that activin may play a part in increasing sensitivity of granulosa cells to FSH, which is necessary for continued growth of the follicle when FSH levels begin to fall (Baird, 1987; Baird, 1991). Activin may also act as an endocrine hormone, since it is capable of stimulating FSH secretion (Schwall *et al.* 1988; Rivier & Vale, 1991).

The study of the synthesis and secretion of inhibins and activins is extremely complex, and it is not yet clear which active molecules are secreted by granulosa cells, and under what circumstances. At the time of the experiments described in this chapter, the cDNA encoding all three inhibin/activin subunits became available (Esch *et al.* 1987), and it became possible to study the regulation of production of these hormones at the level of their mRNA. Data on the levels of mRNA encoding the three subunits cannot be directly extrapolated to the level of protein secretion, but this approach provides a simpler model for understanding the regulation of expression of inhibins and activins.

When these experiments were carried out it was known that inhibin secretion by cultured granulosa cells is stimulated by FSH (Bicsak *et al.* 1986; Suzuki *et al.* 1987; Zhang *et al.* 1987b; Bicsak *et al.* 1988). FSH had also been shown to stimulate expression of inhibin α-subunit mRNA (Woodruff *et al.* 1987), but nothing was known about the regulation of expression of β-subunit mRNA, other than the way in which their levels

changed during the oestrous cycle (Meunier *et al.* 1988a; Woodruff *et al.* 1988). A considerable amount of work had also been published on the effects of growth factors on immunoreactive inhibin synthesis and secretion by cultured granulosa cells (Suzuki *et al.* 1987; Zhang *et al.* 1987b; Zhang *et al.* 1988a; Bicsak *et al.* 1986; Franchimont *et al.* 1986), but little was known about the effects of steroids. One study showed a stimulation of bovine granulosa cell secretion of inhibin bioactivity by androgens in the absence of FSH (Henderson & Franchimont, 1983), although these cells were highly differentiated, and this observation has not been confirmed in other species. Therefore the main objectives of the experiments described in this chapter were to characterise the way in which all three genes are regulated by gonadotrophins both *in vivo* and *in vitro*, and to study the effects of androgens and oestrogens on inhibin subunit gene expression, alone and in the presence of FSH. In order to understand the role of inhibins and activins in the paracrine control of follicle development, it is important to know at what stage of differentiation the expression of these genes is activated. Therefore, levels of mRNA encoding two other markers of granulosa cell differentiation, LH receptor and P450arom, and a marker of luteal function, P450scc, were also measured, for comparison with the inhibin subunits.

2 Materials and Methods

All materials and methods used in the experiments described in this chapter were as described in chapter 2, section 2, except for the following additional materials and methods.

2.1 Inhibin Radioimmunoassay

Inhibin secreted into the culture medium was measured by specific radioimmunoassay (Sharpe *et al.* 1988) using a rabbit antiserum against a synthetic inhibin α-subunit N-terminal 26 amino acid peptide ($pI\alpha^{1-26}$-Gly^{27}-Tyr^{28}), synthesised and supplied by Dr.J.Rivier, The Salk Institute, La Jolla, CA, U.S.A. This peptide was also used as the standard and was radioiodinated by Ms W. Crow, by the chloramine T method, for use as

the tracer. The tracer had a specific activity of 250-350mCi/mg. The antibody (R150) was used in the assay at a final dilution of 1:100,000 in assay buffer (PBS, containing 0.1% BSA), at which concentration it specifically bound 26-39% of the added tracer. A standard curve of 1.9-1000pg pIα^{1-26}-Gly^{27}-Tyr^{28} was used. The sensitivity of the assay was in the range 5-9pg pIα^{1-26}-Gly^{27}-Tyr^{28}. Samples and standards were incubated overnight at 4C in the presence of antibody before tracer ($1.5x10^4$cpm) was added. Tubes were incubated overnight at 4C again before addition of donkey anti-rabbit serum and normal rabbit serum (at dilutions of 1:40 and 1:500, respectively, SAPU), to precipitate bound hormone. Tubes were incubated overnight again, 1ml saline was added to all tubes, and tubes were centrifuged at 3000rpm for 30min. Supernatants were discarded and precipitated radioactivity was measured using a gamma counter (MultiGamma, LKB Wallac). Standard curves were calculated using commercial software (AssayZap, BioSoft, Cambridge, UK). Culture media from individual wells (10µl) were assayed in duplicate, and results were expressed as ng of pIα^{1-26}-Gly^{27}-Tyr^{28} equivalent per 10^6 viable cells in the original inoculum per 48h. Inter- and intra-assay variation were 18% and 8%, respectively.

Statistical analysis was carried out using commercial software (CLR ANOVA, Clear Lake Research Inc., Houston, TX, USA). Analysis of variance was carried out on results after log transformation to reduce heterogeneity of variation, and statistical differences at the $p<0.05$ level were revealed using Duncan's Multiple Range Test.

2.2 cDNA Probes

cDNA clones encoding all three rat inhibin subunits were obtained from Dr.S.Shimasaki, Department of Molecular Endocrinology, Whittier Institute, La Jolla, California, USA. These cDNA clones (α7, β_A30 and β_B11 (Esch *et al.* 1987)) all contained ~1.5kb inserts in the *Eco*RI site of the plasmid pUC18.

The cDNA encoding the bovine P450scc enzyme was obtained from Dr.E.R.Simpson, Department of Obstetrics and Gynaecology, University of Texas Southwestern Medical Centre, Dallas, Texas, USA (John *et al.* 1984).

The ~1.7kb insert was inserted in the *Pvu*II site of pBR322. In Northern hybridisation, the cDNA was found to cross-hybridise with rat and human P450scc mRNA.

The cDNA encoding the rat aromatase enzyme was obtained from Dr.J.S.Richards, Department of Cell Biology, Baylor College of Medicine, Houston, Texas, USA (Hickey *et al.* 1990). The clone used contained an insert approximately 1.2kb in length, cloned into the *Eco*RI site of pGEM3.

A 0.6kb cDNA encoding the rat LH receptor, inserted in the *Eco*RI site of pGEM3, was obtained from Dr.A.J.W.Hsueh, Division of Reproductive Biology, Department of Obstetrics and Gynaecology, Stanford University Medical Centre, Stanford, California, USA (LaPolt *et al.* 1990a).

All cDNAs were excised from the parent plasmid using *Eco*RI in all cases except the bovine P450scc cDNA which was liberated with *Pvu*II. Inserts were purified by gel electrophoresis, and labelled by random priming as described in chapter 2.

2.3 Slot-Blot Analysis of RNA

Where only very small amounts (<5μg) of total RNA were available for analysis, mRNA encoding inhibin α-subunit mRNA was measured by slot-blot analysis, using a commercial slot-blot manifold (Bio-Dot SF apparatus, Bio Rad) according to the manufacturers' instructions. Total RNA was extracted from cultured granulosa cells, and its concentration determined by the methods described in chapter 2. Equal amounts of total RNA were dissolved in 500μl of an ice-cold solution of 10mM NaOH and 1mM EDTA immediately before being applied to individual slots of the apparatus, and blotted to a Zeta-Probe (Bio Rad) charged nylon membrane by vacuum, until all wells were dry. A further 500μl of 10mM NaOH and 1mM EDTA was added to each well, and vacuum was applied once more, until all wells were dry. The manifold was dismantled and the blot was rinsed in 2xSSC, 0.1% SDS, before immediate probing for inhibin α-subunit mRNA. Prehybridisation, hybridisation and washing were as described for DNA probes in chapter 2.

2.4 Stripping and Reprobing Northern Blots

After probing a blot for an mRNA of interest, it was often necessary to hybridise the same blot with other probes. Before hybridisation with subsequent probes, probes were removed from membranes by pouring a boiling solution of 0.1% SDS onto the RNA-bearing face of the membranes, and allowing the solution to cool to room temperature. The process was repeated at least twice. Blots were exposed to x-ray film for at least 3 days to confirm removal of probes. Blots were then prehybridised and hybridised as described in chapter 2.

3 Results

3.1 Effects of Gonadotrophins on Inhibin Gene Expression *In Vivo*

Four inhibin subunit mRNA transcripts were detected by Northern analysis of granulosa cell total RNA (Fig.4.1). One band of approximately 1.7kb was detected using the inhibin-α cDNA, one band of ~6.5kb was detected using the inhibin-β_A cDNA, and two bands of ~4.4 and ~3.4kb, of which the larger was the more abundant, hybridised to the inhibin-β_B cDNA probe, in agreement with previous reports (Esch *et al.* 1987; Feng *et al.* 1989a). The effects of FSH and hCG on the expression of the three inhibin subunit genes *in vivo* were examined, and the results from two experiments are presented in Fig.4.2. Granulosa cells from control animals contained high levels of inhibin α-subunit mRNA, and moderate levels of inhibin β_B-subunit mRNA, but very low or undetectable levels of inhibin β_A-subunit mRNA. Injection of FSH (20μg oFSH, every 12h for two days prior to removal of ovaries) caused a small increase in levels of inhibin α-subunit mRNA, and a more pronounced increase in the expression of mRNA encoding the two β-subunits. One injection of 20IU hCG 12h before the animals were killed also caused a marked increase in levels of β_A- and β_B-subunit mRNA, and a small increase in α-subunit mRNA. However, injection of 20IU hCG after treatment of animals with FSH caused a reduction in the levels of

Fig.4.1. Northern blot analysis of inhibin subunit mRNA in rat granulosa cells. Cells were cultured for 48h in the presence of 100ng/ml FSH. Total RNA (5μg) was subjected to electrophoresis in an agarose-formaldehyde gel, and transferred to a nylon membrane. The membrane was sequentially probed with ^{32}P-labelled rat inhibin subunit cDNA probes as marked. Hybridisation was carried out overnight at 65C, and washing was carried out for 2x30min at 65C. Blots were exposed to x-ray film at -70C for the following times: α-subunit, 24h; β_A-subunit, 8 days; β_B-subunit, 50h. Positions of the migration of RNA molecular weight markers are indicated on the left.

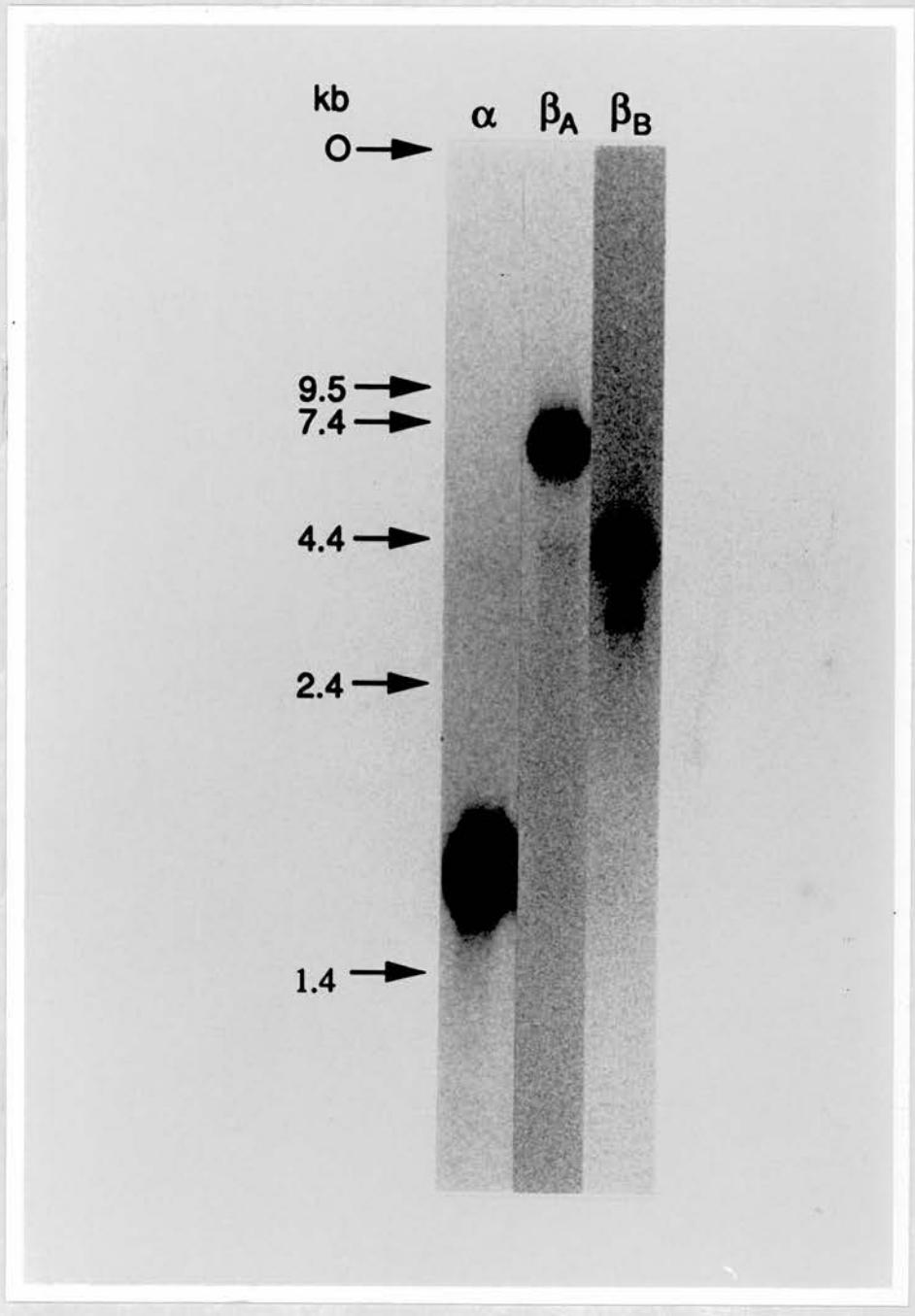
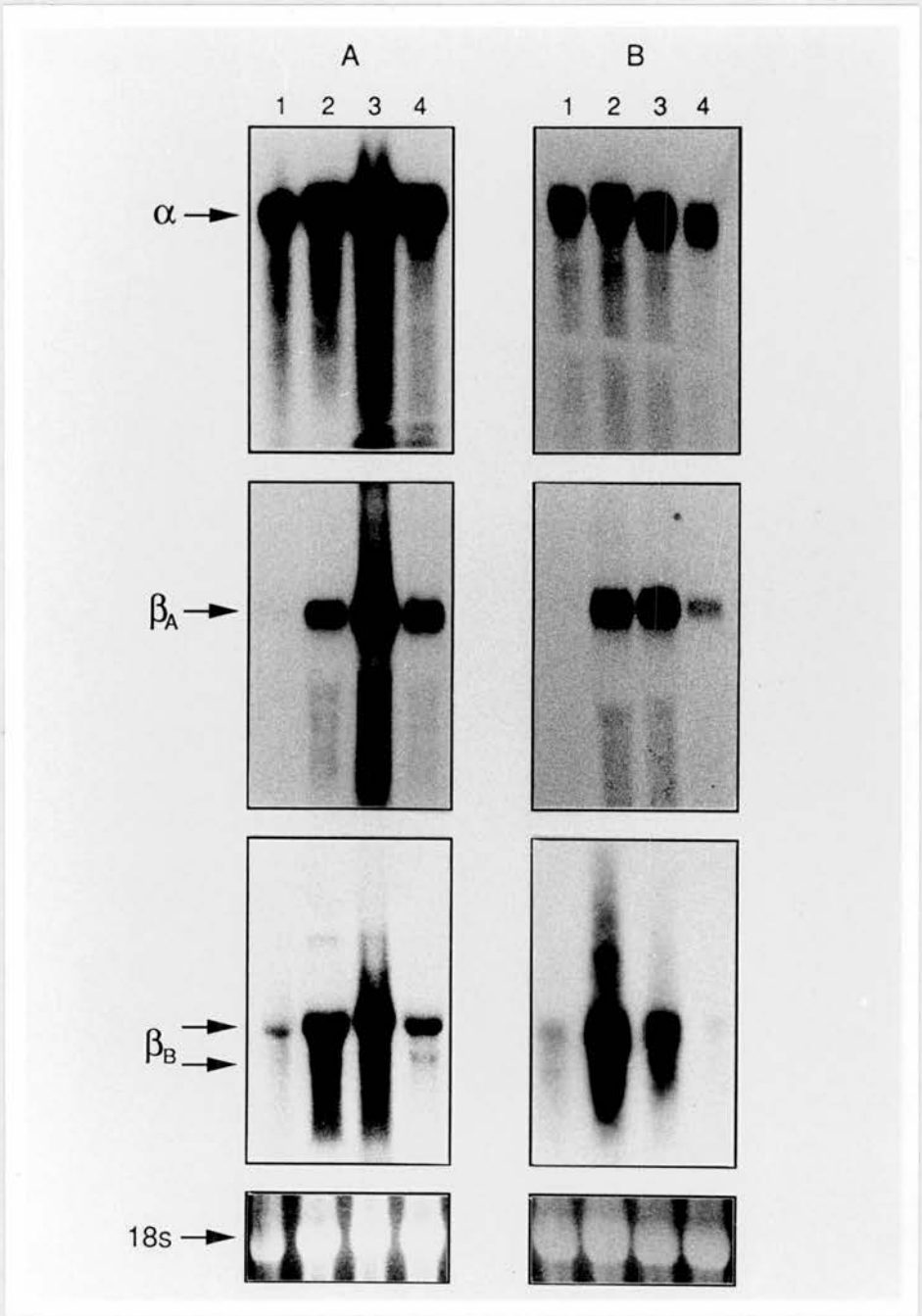

kb
α
β_A
β_B
O
9.5
7.4
4.4
2.4
1.4

Fig.4.2. Regulation of rat granulosa cell inhibin subunit mRNA expression by gonadotrophins *in vivo*. Immature female rats were treated for four days with DES, and 1) no further treatment (control), 2) one injection of 20IU hCG 12h before isolation of granulosa cells, 3) four injections of 20μg oFSH every 12h for 48h before isolation of cells, and 4) four injections of 20μg oFSH every 12h for 48h, plus one injection of 20IU hCG 12h before isolation of cells. Panels A and B show the results from two separate experiments. Granulosa cells were isolated, and total RNA was extracted immediately. Twenty micrograms of total RNA from each treatment were subjected to electrophoresis in agarose-formaldehyde gels, and transferred to nylon membranes, which were sequentially probed with ^{32}P-labelled rat inhibin subunit cDNA probes as marked. Autoradiograms from experiment A were exposed for 5h (α), 48h (β_A) and 16h (β_B), and those from experiment B were exposed for 9h (α), 20h (β_A) and 12h (β_B). The 18s ribosomal RNA bands, stained with ethidium bromide, from the gel used to generate each blot are shown at the bottom to indicate loading of RNA.


A
B
1 2 3 4
1 2 3 4
α
β_A
β_B
18s

mRNA encoding all three inhibin subunits, when compared to treatment with FSH alone. The levels of mRNA encoding the β-subunits appeared to be more strongly influenced by FSH and hCG than the α-subunit mRNA levels.

The relationship between the regulation of the expression of the inhibin subunit genes and other markers of granulosa cell differentiated function was investigated by probing the same samples for mRNA encoding P450scc, P450arom and the LH receptor.

At least three different transcripts present in total RNA from ovarian cells hybridised to the LH receptor cDNA probe (Fig.4.3). The most abundant transcript was approximately 6.5kb in length, with much less intense bands at approximately 4.2, 2.7 and 1.4kb, in agreement with previous reports (LaPolt *et al.* 1990a; Segaloff *et al.* 1990). mRNA encoding the LH receptor was not detected in granulosa cells from control animals. In one experiment, injection of hCG alone caused a small increase in levels of LH receptor mRNA (Fig.4.3B), although in another no effect of hCG alone was seen (Fig.4.3A). FSH caused a massive induction of granulosa cell LH receptor mRNA, which was strongly inhibited by subsequent treatment with hCG. For comparison, RNA extracted from ovaries from which granulosa cells had been isolated (residual ovary), and which therefore contained the thecal and interstital cells, was also probed for LH receptor mRNA (Fig.4.3C). It was found that RNA extracted from residual ovaries of control animals contained levels of LH receptor mRNA lower than, but comparable to the levels found in the granulosa cells of FSH-treated animals. Levels of LH receptor mRNA in residual ovary samples were unaffected by treatment of animals with FSH, but were reduced to undetectable levels within 12h of injection of 20IU hCG, with or without concomitant FSH treatment.

The aromatase cDNA hybridized to three major bands in granulosa cell RNA, the predominant one being approximately 2.7kb in size, with two rather less intense bands migrating at approximately 2.1 and 1.5kb (Fig.4.4). Other studies have suggested that the 2.7kb species is the functional aromatase mRNA, and that the other transcripts lack the region encoding the haem-binding domain of the enzyme (Lephart *et al.* 1990). P450arom mRNA was undetectable in control granulosa cell RNA

Fig.4.3. Regulation of rat granulosa cell LH receptor mRNA expression by gonadotrophins *in vivo*. Animals were treated as described in the legend to Fig.4.2. Panels A and B show the results from the same experiments presented in Fig.4.2. Panel C shows expression of LH receptor mRNA in residual ovarian tissue under the same experimental treatments. Granulosa cells were isolated, and total RNA was extracted immediately from granulosa cells and residual ovarian tissue. Twenty micrograms of total RNA from each treatment were subjected to electrophoresis in agarose-formaldehyde gels, and transferred to nylon membranes, which were probed with a ^{32}P-labelled rat LH receptor cDNA probe. Autoradiograms were exposed for 18h. Migration of 18s and 28s ribosomal RNA is indicated. Markers on the left hand side refer to panel A, and those on the right refer to panels B and C. The 18s ribosomal RNA bands, stained with ethidium bromide, from the gel used to generate each blot are shown at the bottom to indicate loading of RNA.

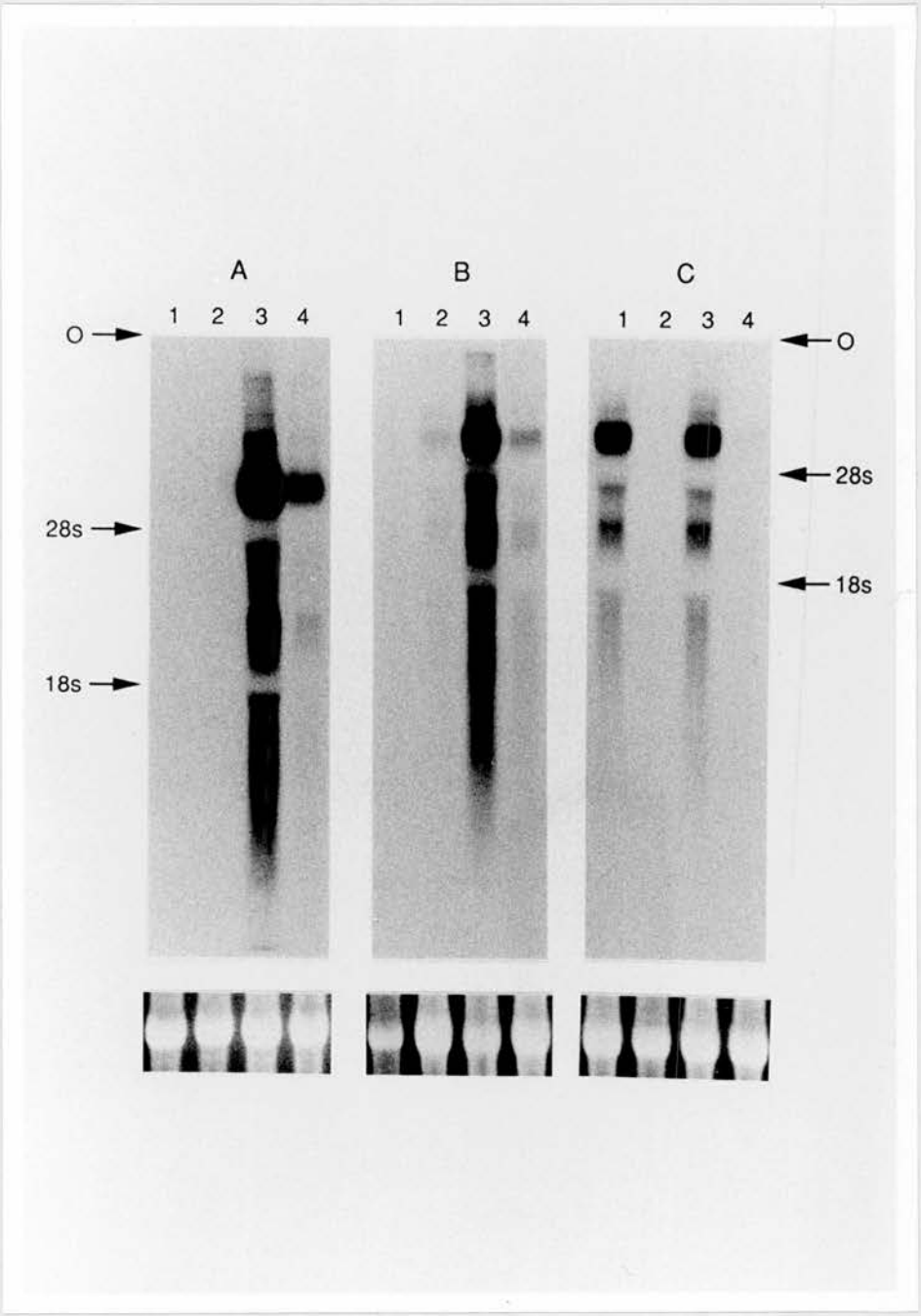
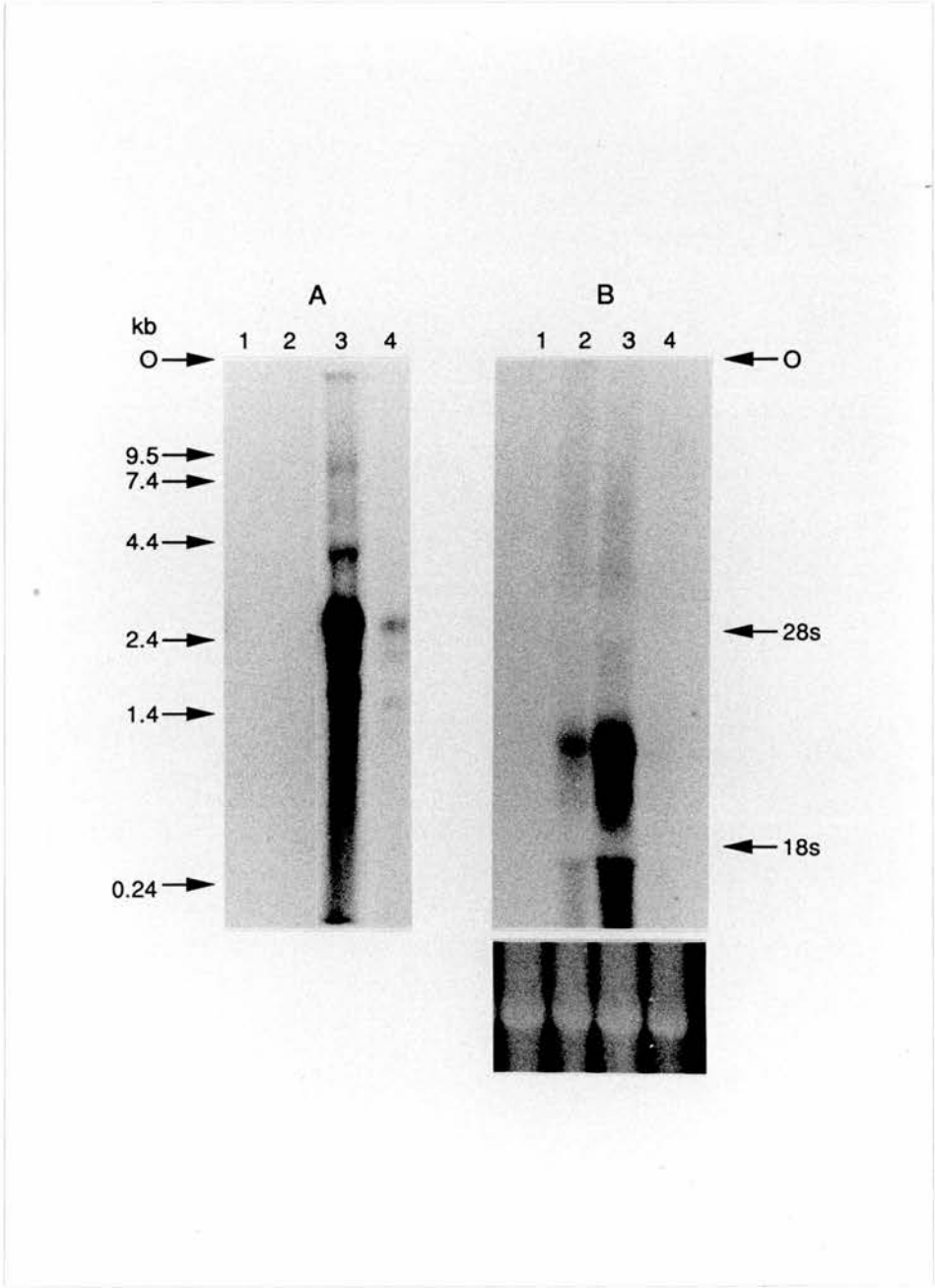

A
B
C
1 2 3 4
1 2 3 4
1 2 3 4
O
28s
18s
O
28s
18s

Fig.4.4. Regulation of rat granulosa cell P450arom mRNA expression by gonadotrophins *in vivo*. Animals were treated as described in the legend to Fig.4.2. Panels A and B show the results from the same experiments presented in Figs.4.2 and 4.3. Granulosa cells were isolated, and total RNA was extracted immediately. Twenty micrograms of total RNA from each treatment were subjected to electrophoresis in agarose-formaldehyde gels, and transferred to nylon membranes, which were probed with a ^{32}P-labelled rat P450arom cDNA probe. Autoradiograms were exposed for 4 days. Migration of RNA molecular weight markers (panel A) or of 18s and 28s ribosomal RNA (panel B) are indicated. The 18s ribosomal RNA bands, stained with ethidium bromide, from the gel used to generate blot B are shown at the bottom to indicate loading of RNA.


A
B
kb
1 2 3 4
1 2 3 4
O
9.5
7.4
4.4
2.4
1.4
0.24
O
28s
18s

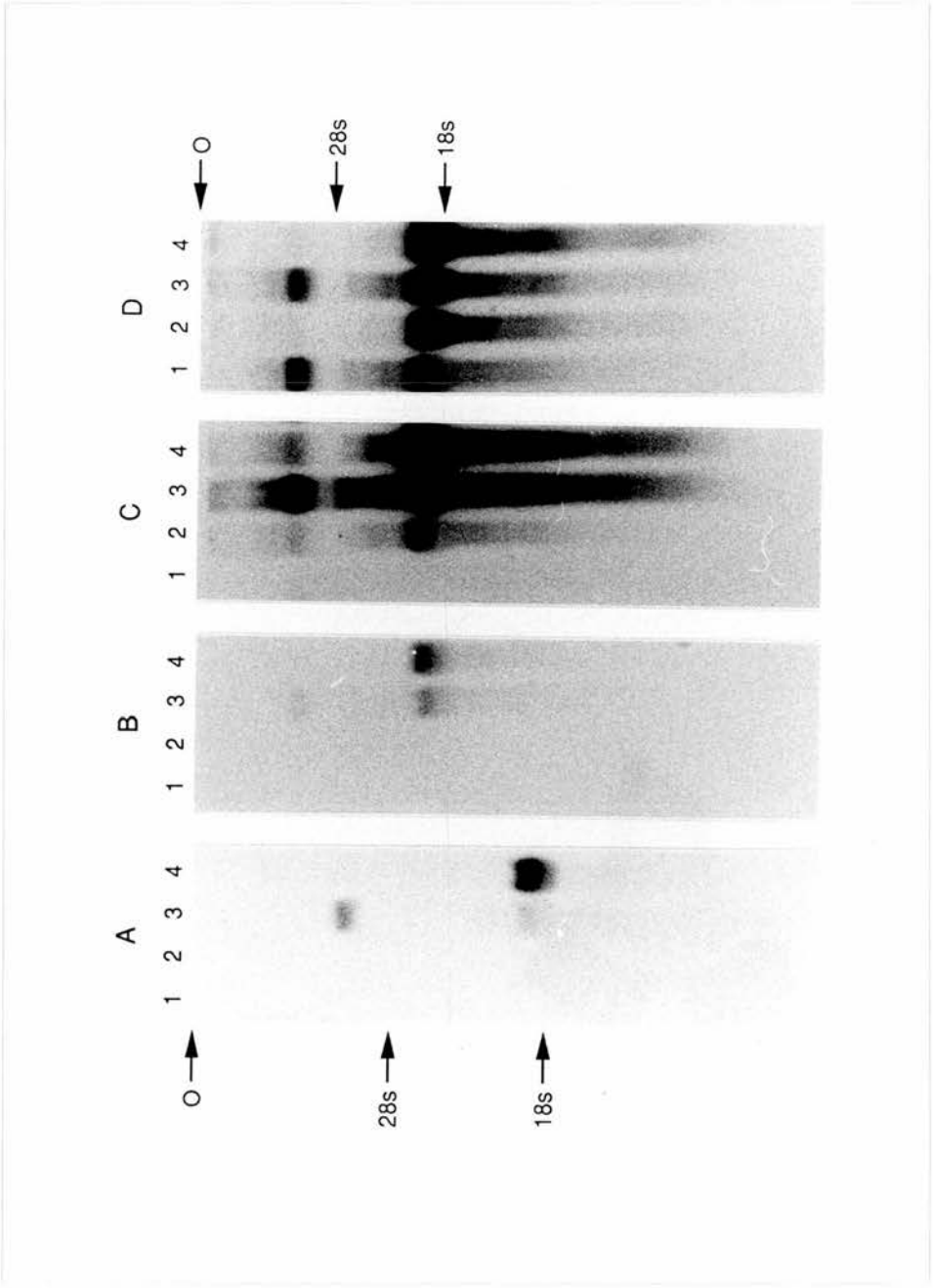
samples. hCG caused a slight increase in levels of P450arom mRNA only in one experiment (4.4B), and it was this experiment in which hCG caused an increase in LH receptor mRNA. FSH caused a dramatic increase in levels of mRNA encoding P450arom, and injection of hCG following FSH treatment resulted in a precipitous drop in P450arom mRNA levels.

Expression of P450scc, however, was regulated in a significantly different fashion from the inhibin subunits, LH receptor and P450arom. A single transcript was detected with the P450scc cDNA probe (Fig.4.5), as a broad band approximately 2.1kb in size, in agreement with other studies (Goldring *et al.* 1987; Trzeciak *et al.* 1987). No mRNA encoding P450scc was detected in RNA samples from granulosa cells isolated from control animals. Again, hCG treatment in the absence of FSH had variable effects on levels of P450scc mRNA. Only in the experiment in which hCG stimulated expression of mRNA encoding P450arom and LH receptor were P450scc mRNA levels also increased by hCG treatment (Fig.4.5C). The effect of hCG alone, where it was observed, was small in comparison to the potent stimulation of P450scc levels by FSH. However, in contrast to levels of mRNA encoding the inhibin subunits, LH receptor and P450arom, the levels of P450scc mRNA were further increased by injection of hCG following FSH treatment. Levels of P450scc mRNA in residual ovarian tissue from control animals were found to be much higher than those found in control granulosa cells (Fig.4.5D). FSH treatment did not affect levels of P450scc mRNA in residual ovary. However, hCG, alone or in combination with FSH, caused an increase in levels of P450scc mRNA.

3.2 Effects of Gonadotrophins on Inhibin Gene Expression *In Vitro*

Initially, the *in vitro* effects of FSH and hCG on inhibin subunit gene expression were studied in experiments analogous to those described above. Granulosa cells were isolated from immature rats which had been treated *in vivo* with DES for four days. These cells were cultured for 48h in the presence or absence of 100ng/ml hFSH. Cells which received no hormones during the first 48h of culture were

Fig.4.5. Regulation of rat granulosa cell P450scc mRNA expression by gonadotrophins *in vivo*. Animals were treated as described in the legend to Fig.4.2. Granulosa cells were isolated, and total RNA was extracted immediately. Twenty micrograms of total RNA from each treatment were subjected to electrophoresis in agarose-formaldehyde gels, and transferred to nylon membranes, which were probed with a ^{32}P-labelled bovine P450scc cDNA probe. Panels A and B show the results from the same experiments presented in Figs.4.2, 4.3 and 4.4, and both were exposed for 16h. Panel C shows a longer exposure (19 days) of the Northern blot shown in panel B, and panel D shows expression of P450scc in residual ovarian tissue under the same experimental conditions (exposure: 19 days). Migration of 18s and 28s ribosomal RNA is indicated. Markers on the left hand side refer to panel A, and those on the right refer to panels B-D. The high molecular weight band visible on the autoradiographs is due to LH receptor probe which was incompletely stripped from the membrane.


O
28s
18s
D
C
B
A
1 2 3 4
O
28s
18s

cultured for a further 24h in the presence or absence of 200mIU/ml hCG. Cells which received FSH during the first 48h of culture were cultured for a further 24h in the presence of 100ng/ml FSH, in the presence or absence of 200mIU/ml hCG.

The results of a representative experiment are shown in Fig.4.6. Low levels of inhibin α- and β_A-subunit mRNA were detected in RNA samples from control granulosa cells. Inhibin β_B-subunit mRNA was undetectable in control cells in the experiment shown. However, the amount of total granulosa cell RNA loaded on the Northern blot which was probed for inhibin β_B-subunit mRNA was much less than that loaded on the blot probed for α- and β_A-subunit mRNA. Treatment of cells with FSH for 72h caused a marked increase in levels of mRNA encoding all three inhibin subunits. In the absence of FSH, incubation with hCG for 24h did not have any effect on levels of mRNA encoding any of the inhibin subunits. However, after pretreatment of cells with FSH for 48h, hCG suppressed levels of mRNA encoding all three inhibin subunits almost to control levels, even though FSH was still present in the culture medium.

The levels of mRNA encoding P450arom, P450scc and LH receptor in these RNA samples were also investigated (Fig.4.7). As was the case *in vivo*, it was found that the regulation of P450arom and LH receptor mRNA levels followed the same pattern as the mRNA encoding the three inhibin subunits. No mRNA encoding these proteins was detected in RNA samples from untreated or hCG-treated cultures. FSH treatment *in vitro* caused an increase in levels of P450arom and LH receptor mRNA, which was inhibited by concomitant treatment with hCG. Again, P450scc mRNA levels were differently regulated by gonadotrophins, being stimulated moderately by FSH treatment, and more strongly by hCG following FSH treatment.

The molecular weights of mRNA species encoding inhibin subunits, P450scc, P450arom and LH receptor detected in RNA from cultured cells were identical to those detected in RNA from freshly isolated cells.

The time-course of these effects of FSH was studied. Cells were cultured for 24h in the absence of hormones, to permit attachment. Cells were then treated with FSH (100ng/ml). RNA was extracted from the cells

Fig.4.6. Regulation of inhibin subunit mRNA expression in rat granulosa cells by gonadotrophins *in vitro*. Granulosa cells were cultured for 48h in the presence (lanes 3 and 4) or absence (lanes 1 and 2) of 100ng/ml FSH. Medium was changed, and cells were cultured for a further 24h in the absence of hormones (lane 1), or in the presence of 200mIU/ml hCG (lane 2), 100ng/ml FSH (lane 3), or 200mIU/ml hCG plus 100ng/ml FSH (lane 4). RNA was extracted from monolayers, and 20μg (left hand panels) or 4μg (right hand panels) of total RNA were subjected to electrophoresis in agarose-formaldehyde gels. RNA was transferred to nylon membranes, which were hybridised with ^{32}P-labelled rat inhibin subunit cDNA probes as marked. Exposure times were: α, 9h; β_A, 17h; β_B, 12 days. The 18s ribosomal RNA bands, stained with ethidium bromide, from the gels used to generate the blots are shown at the bottom to indicate loading of RNA.

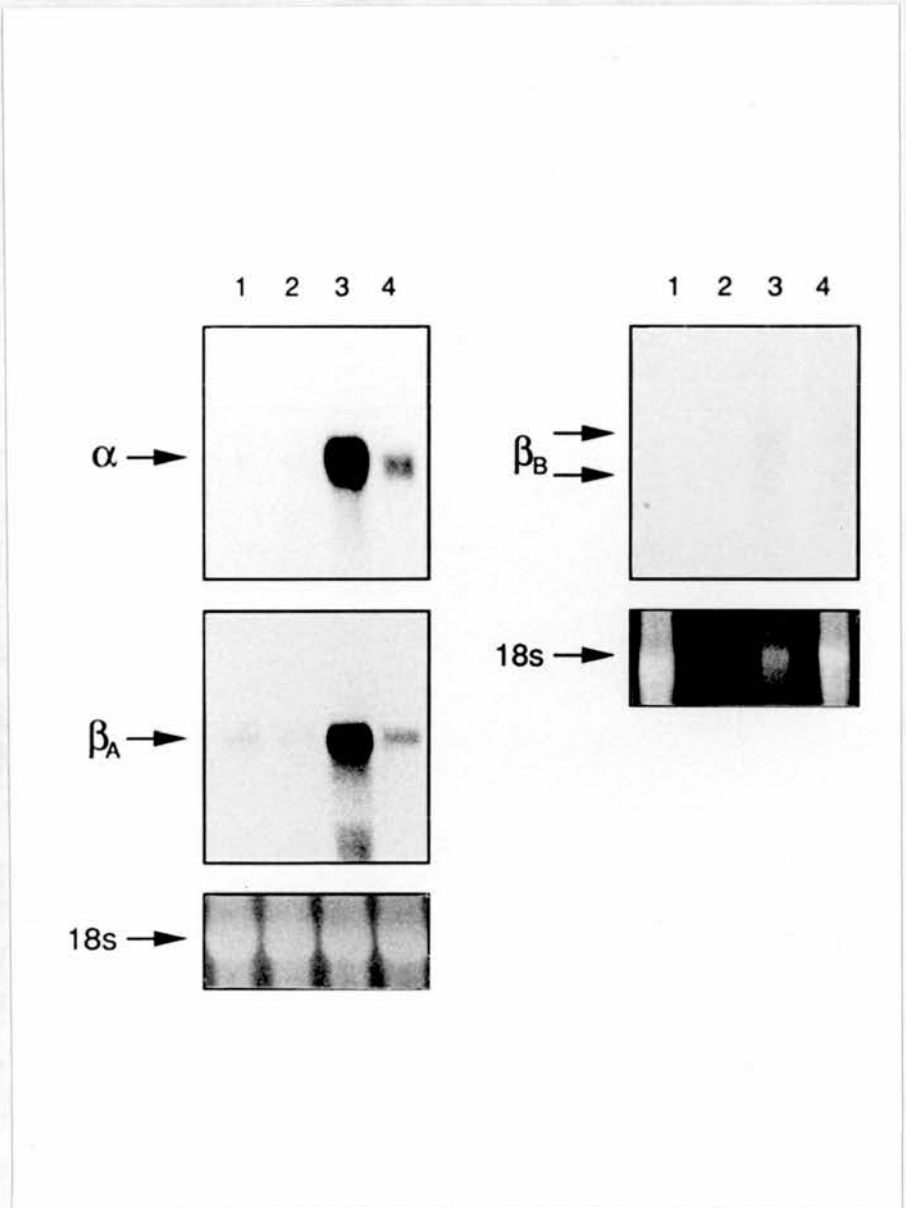
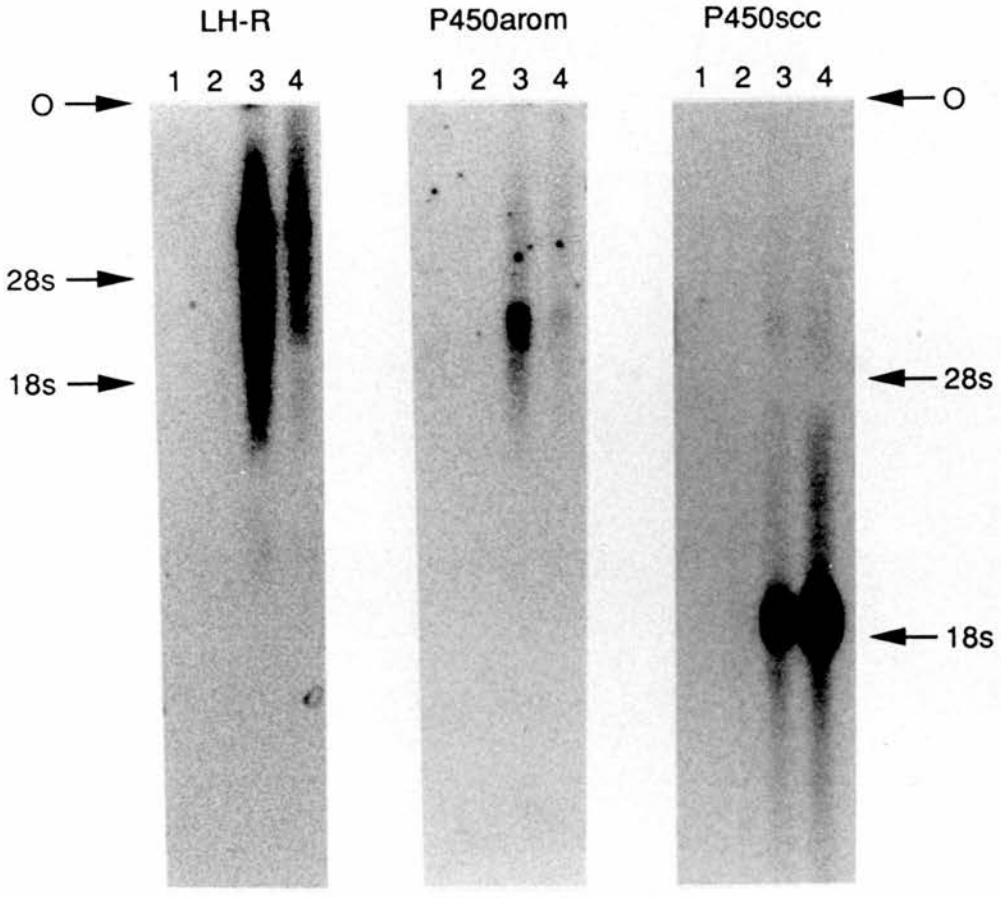

1 2 3 4
α
βA
18s
1 2 3 4
βB
18s

Fig.4.7. Regulation of expression of mRNA encoding LH receptor, P450arom and P450scc in rat granulosa cells by gonadotrophins *in vitro*. Cells were treated as described in the legend to Fig.4.7. RNA was extracted from monolayers, and 6μg (left and centre panels) or 10μg (right panel) of total RNA were subjected to electrophoresis in agarose-formaldehyde gels. RNA was transferred to nylon membranes, which were hybridised with ^{32}P-labelled rat LH receptor (LH-R), rat P450arom and bovine P450scc cDNA probes as marked. Exposure times were: left and centre, 13 days; right, 20 days. Migration of 18s and 28s ribosomal RNA is indicated. Markers on the left hand side refer to panels A and B, and those on the right refer to panel C.


LH-R
P450arom
P450scc
1 2 3 4
1 2 3 4
1 2 3 4
O
28s
18s
O
28s
18s

at fixed time-points of 0, 1, 2, 4, 8, 24 and 48 hours after treatment. Due to the high abundance of the inhibin α-subunit mRNA, a possible increase in the level of this message could be seen as early as 2h after FSH treatment (Fig.4.8). However, a clear stimulation of inhibin-α mRNA by FSH was not detected until 24h after treatment. Less abundant mRNA species, such as those encoding the inhibin β-subunits (Fig.4.8), LH receptor, P450arom and P450scc (Fig.4.9) were not observed to increase in response to FSH until 24h after treatment. After 48h, the stimulation by FSH of levels of all mRNA species studied was very clear, and so further experiments were conducted for 48h.

3.3 Effects of FSH and Steroids on Inhibin Gene Expression *In Vitro*

Possible modulatory effects of sex steroids on the regulation of inhibin subunit gene expression by FSH were then studied. It was found that, in the absence of steroids, FSH is capable of stimulating expression of mRNA encoding all three inhibin subunits. However, the magnitude of this effect was very variable (Figs.4.10-4.12). In experiments in which 100ng/ml FSH caused a great increase in levels of inhibin subunit mRNA (Figs.4.10 and 4.11), simultaneous treatment with oestradiol or DHT had no further effect. However, in experiments in which FSH, at a dose of 100ng/ml, caused only a slight increase in expression of inhibin subunit mRNA (Fig.4.12), this effect was augmented by androgens and oestradiol at a dose of 10^{-6}M. In such experiments, oestradiol appeared to be more potent than DHT in augmenting this effect of FSH.

In the absence of FSH, no effect of androgen on the levels of inhibin subunit mRNA was observed. However, oestradiol-17β alone, at a concentration of 10^{-6}M, stimulated expression of inhibin α- and β_B-subunit mRNA, without having any measurable effect on levels of mRNA encoding the inhibin β_A-subunit.

The influence of treatment of animals *in vivo* with DES on the regulation of inhibin gene expression *in vitro* was also studied (Fig.4.12). The stimulation of inhibin-α and -β_B mRNA levels by FSH was observed where animals had not been treated with DES. However, no effect of FSH

Fig.4.8. Timecourse of stimulation by FSH of inhibin subunit mRNA expression *in vitro*. Granulosa cells were cultured in the absence of hormones for 24h. Medium was changed and cells were incubated for different times in the presence of 100ng/ml FSH. RNA was extracted from monolayers at the times indicated after FSH addition. Total RNA from each sample (5μg) was subjected to electrophoresis in an agarose-formaldehyde gel, and transferred to a nylon membrane, which were hybridised with ^{32}P-labelled rat inhibin subunit cDNA probes, as marked. Exposure times were: α, 60h; β_A, 19 days; β_B, 7 days. The 18s ribosomal RNA bands, stained with ethidium bromide, from the gel used to generate the blot are shown at the bottom to demonstrate even loading of RNA.

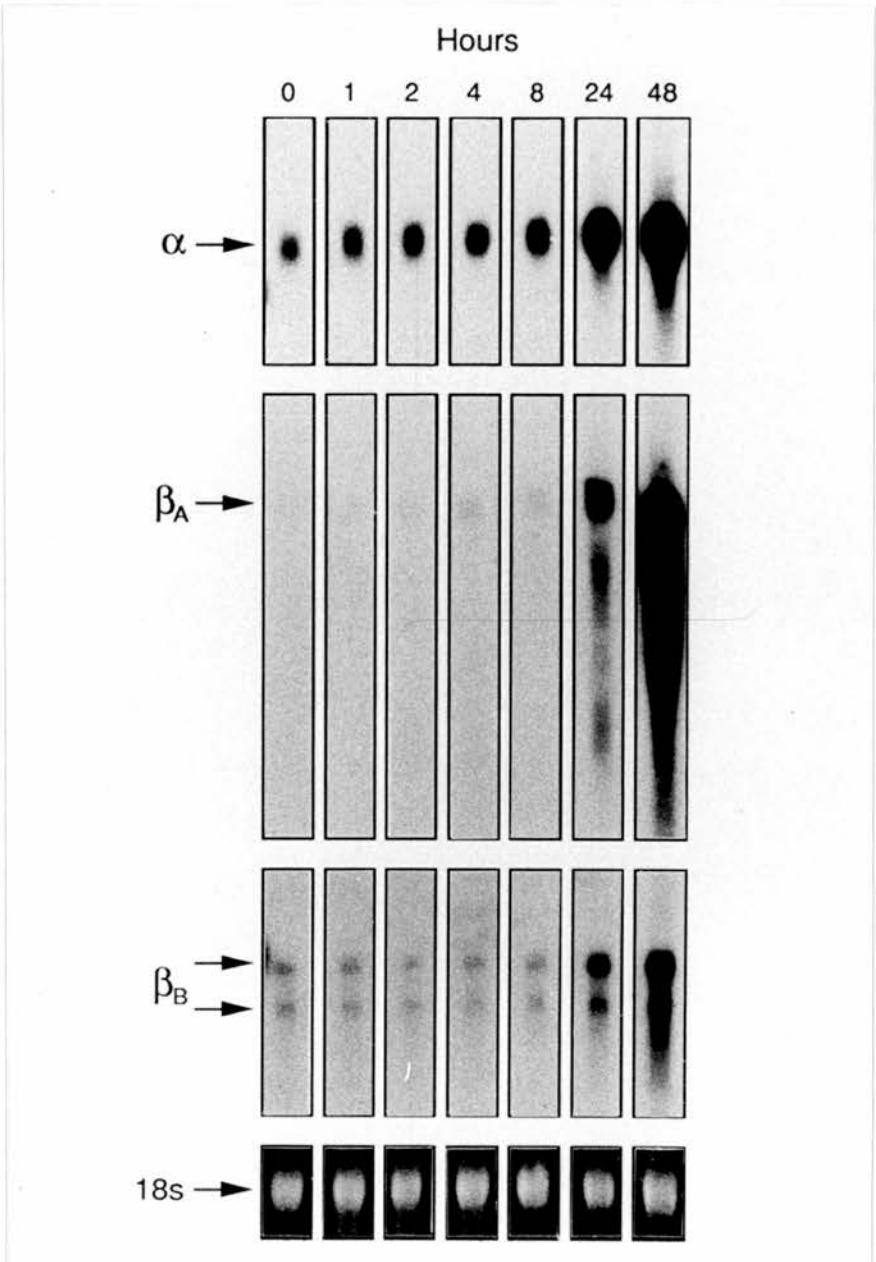

Hours
0
1
2
4
8
24
48
α
βA
βB
18s

Fig.4.9. Timecourse of stimulation by FSH *in vitro* of expression of mRNA encoding LH receptor (top), P450 arom (centre) and P450scc (bottom). Granulosa cells were treated as described in the legend to Fig.4.9. The Northern blot presented here is the same one presented in Fig.4.8. The membrane was sequentially hybridised with ^{32}P-labelled rat LH receptor, rat P450arom and bovine P450scc cDNA probes, as marked. Exposure times were: top, 14 days; centre, 8 days; bottom, 7 days.

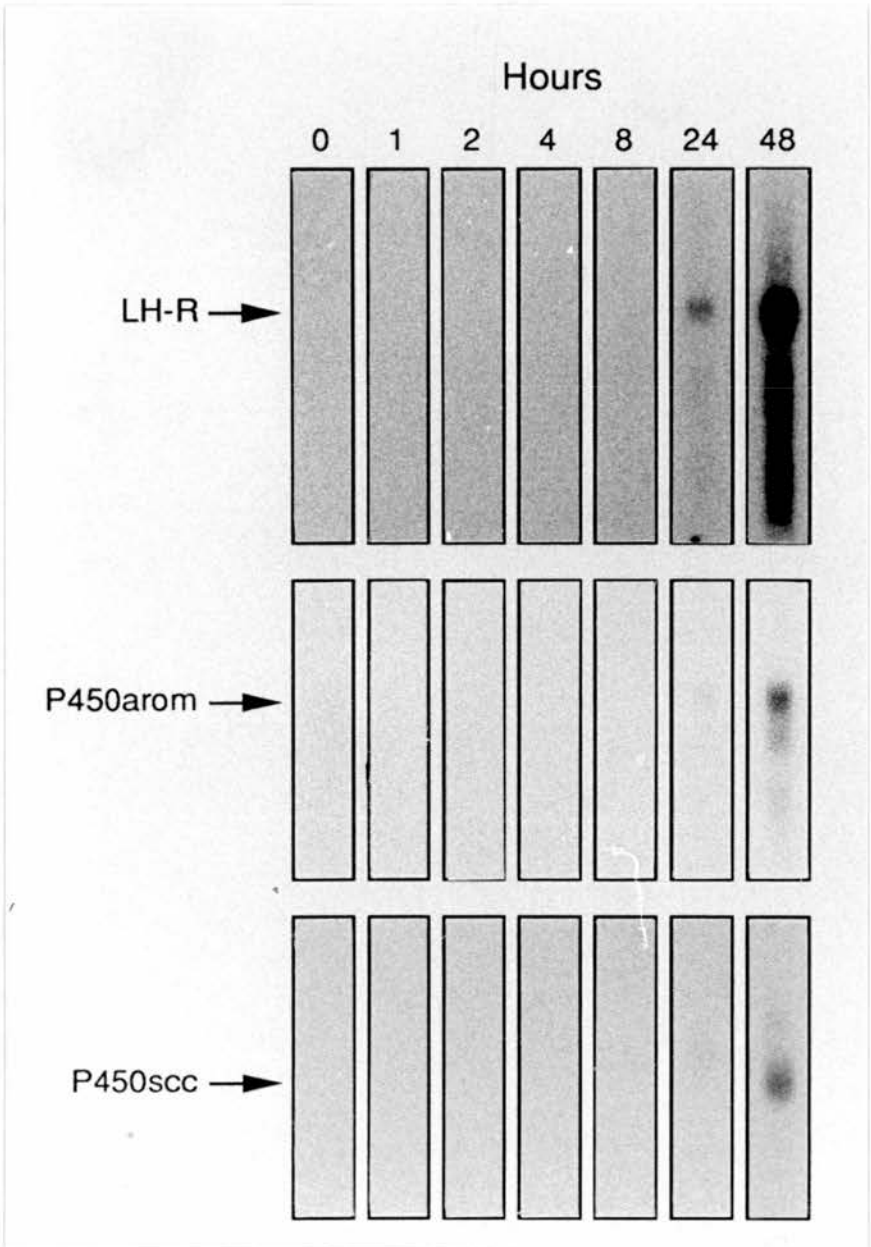

Hours
0
1
2
4
8
24
48
LH-R
P450arom
P450scc

Fig.4.10. Effects of FSH and steroids on expression of inhibin subunit mRNA *in vitro*. Granulosa cells were cultured under the following conditions: 1) control, 2) 10^{-6}M DHT, 3) 10^{-6}M oestradiol, 4) 100ng/ml FSH, 5) 100ng/ml FSH plus 10^{-6}M DHT, and 6) 100ng/ml FSH plus 10^{-6}M oestradiol. RNA was extracted from monolayers and total RNA from each sample (4μg) was subjected to electrophoresis in agarose-formaldehyde gels, and transferred to nylon membranes, which were hybridised with ^{32}P-labelled rat inhibin subunit cDNA probes, as marked. Exposure times were: α, 48h; β_A, 8 days; β_B, 10 days. The 18s ribosomal RNA bands, stained with ethidium bromide, from the gel used to generate the blot are shown at the bottom to demonstrate even loading of RNA.

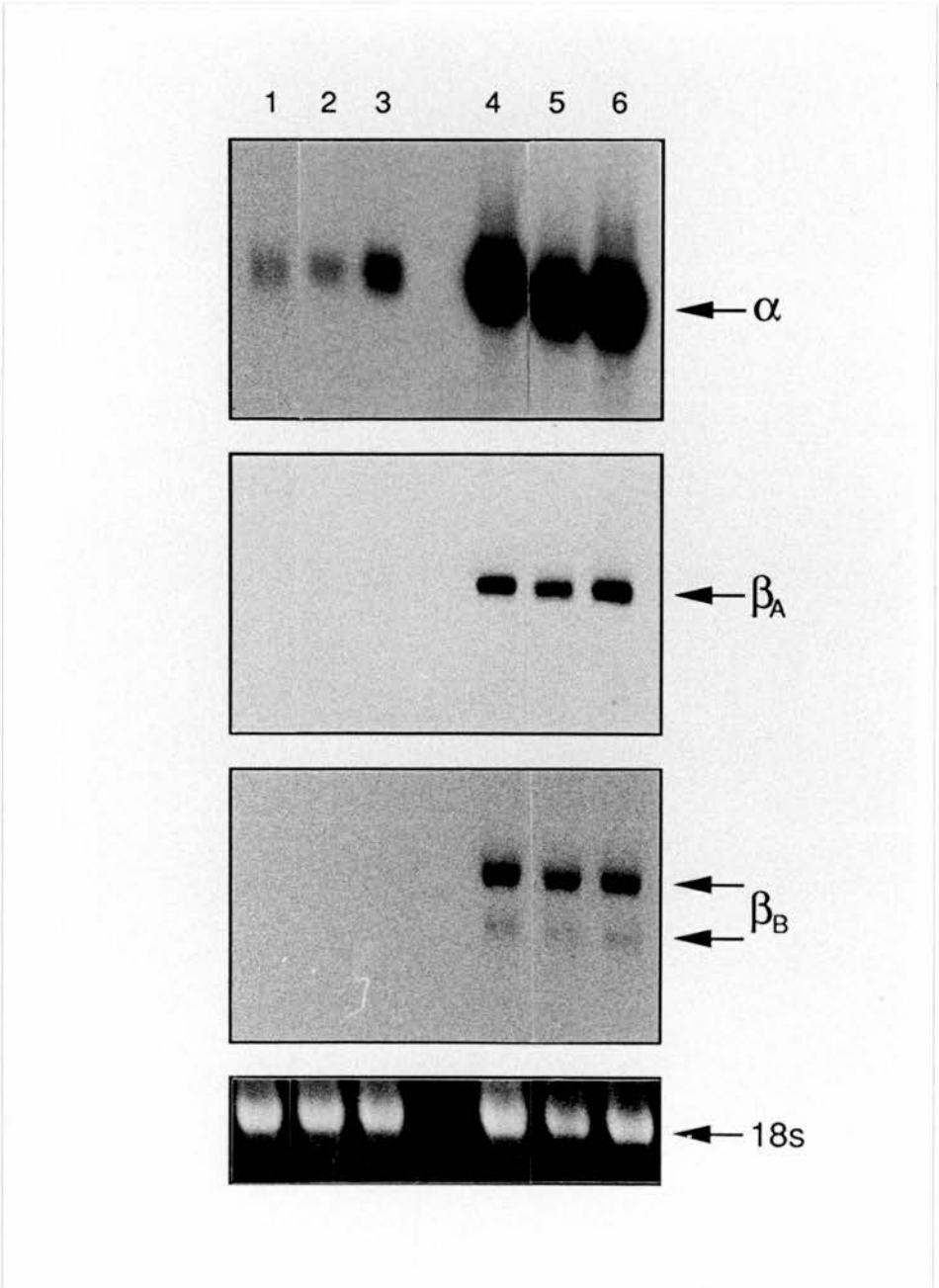

1 2 3 4 5 6
α
β_A
β_B
18s

Fig.4.11. Effects of FSH and steroids on expression of inhibin subunit mRNA *in vitro*. Granulosa cells were cultured under the following conditions: 1) control, 2) 10^{-6}M oestradiol, 3) 10^{-6}M DHT, 4) 100ng/ml FSH, 5) 100ng/ml FSH plus 10^{-6}M oestradiol, and 6) 100ng/ml FSH plus 10^{-6}M DHT. RNA was extracted from monolayers and total RNA from each sample (4μg) was subjected to electrophoresis in agarose-formaldehyde gels, and transferred to nylon membranes, which were hybridised with ^{32}P-labelled rat inhibin subunit cDNA probes, as marked. Exposure times were: α, 24h; β_A, 8 days; β_B, 10 days. The 18s ribosomal RNA bands, stained with ethidium bromide, from the gel used to generate the blot are shown at the bottom to demonstrate even loading of RNA.

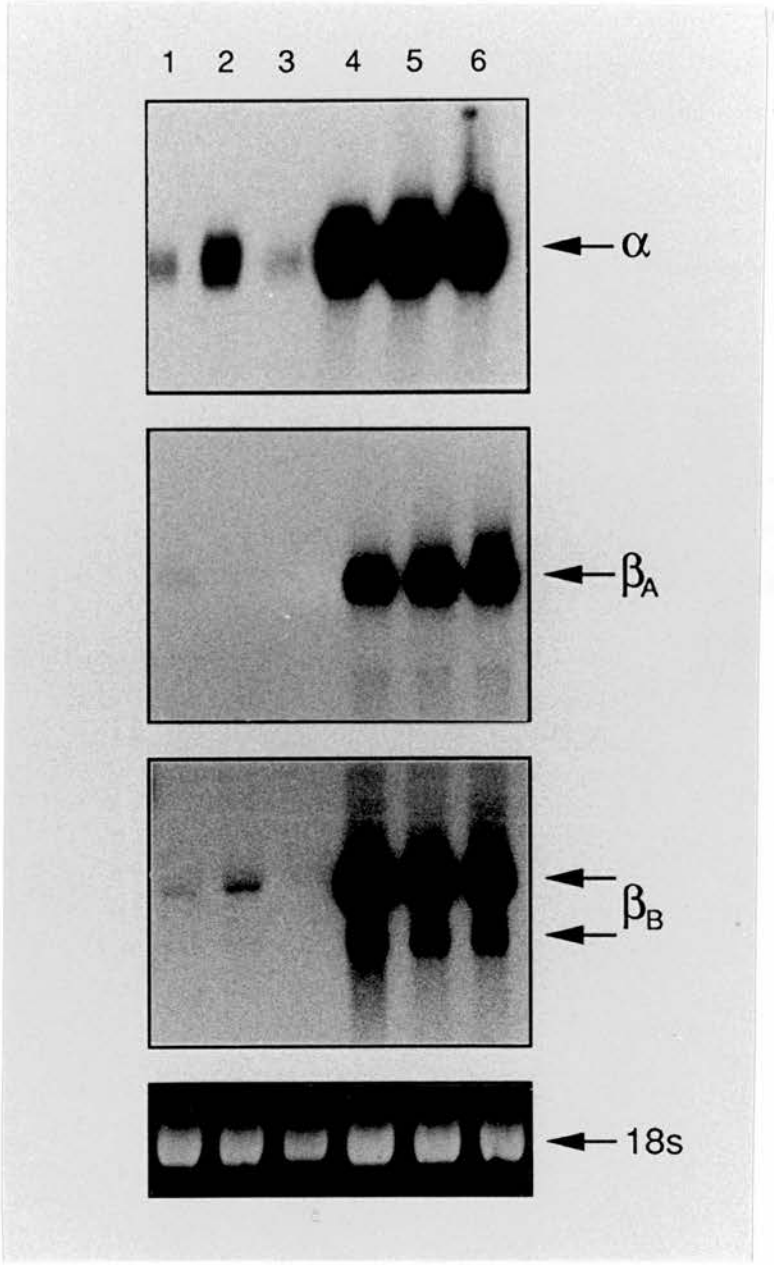
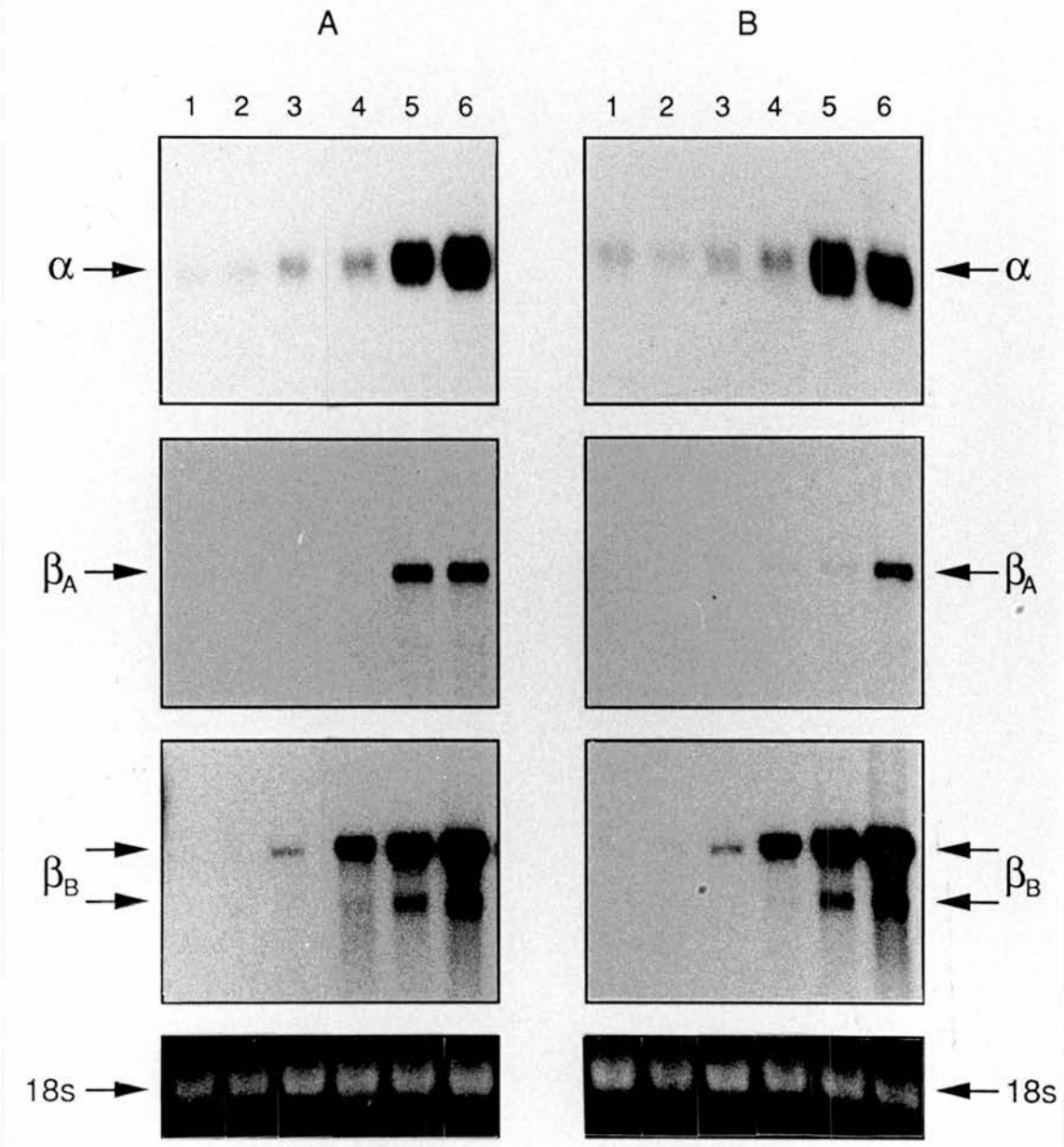

1 2 3 4 5 6
α
β_A
β_B
18s

Fig.4.12. Effects of FSH and steroids on expression of inhibin subunit mRNA *in vitro*. Granulosa cells from animals which were (panel A) and which were not (panel B) treated *in vivo* with DES were cultured under the following conditions: 1) control, 2) 10^{-6}M DHT, 3) 10^{-6}M oestradiol, 4) 100ng/ml FSH, 5) 100ng/ml FSH plus 10^{-6}M DHT, and 6) 100ng/ml FSH plus 10^{-6}M oestradiol. RNA was extracted from monolayers and total RNA from each sample (4μg) was subjected to electrophoresis in agarose-formaldehyde gels, and transferred to nylon membranes, which were hybridised with ^{32}P-labelled rat inhibin subunit cDNA probes, as marked. Exposure times were: α, 20h; β_A, 8 days; β_B, 10 days. The 18s ribosomal RNA bands, stained with ethidium bromide, from the gel used to generate the blot are shown at the bottom to demonstrate even loading of RNA.


A
B
1 2 3 4 5 6
1 2 3 4 5 6
α
α
βA
βA
βB
βB
18s
18s

alone on the expression of β_A-subunit mRNA was observed. The effect of oestradiol in augmenting the effect of FSH on expression of both β-subunit genes was greater than the effect of testosterone regardless of whether animals had been treated with DES. It should be noted, however, that the effect of oestradiol in augmenting FSH-stimulated α-subunit mRNA was no greater than the effect of testosterone in animals which had not been treated with DES. However, this could be explained by a slight difference in loading of RNA on the gel.

Since the effects of sex steroids on the stimulation of inhibin subunit mRNA appeared to depend upon the degree of stimulation by FSH, the effects of steroids on inhibin gene expression were studied in the presence of a lower concentration of FSH (30ng/ml). Fig.4.13 shows that oestradiol is capable of dose-dependently stimulating inhibin α-subunit mRNA levels in the absence of FSH (panel A), and of dose-dependently augmenting the stimulation brought about by FSH at a concentration of 30ng/ml (panel B) or 100ng/ml (panel C). Inhibin β-subunit mRNA levels were not measured in these experiments.

In contrast to oestradiol, DHT was never observed to have any effect on inhibin subunit mRNA levels in the absence of FSH (Fig.4.14). In the presence of 30ng/ml FSH, however, DHT dose-dependently stimulated inhibin α- and β_A-subunit mRNA expression. Basal levels of inhibin-β_A mRNA were unusually high in the experiment shown in Fig.4.14A (compare to control levels in Figs.4.10-4.12), and appeared to be decreased by FSH treatment. Inhibin β_B-subunit mRNA levels were not measured in these experiments.

The regulation of inhibin gene expression by cAMP was also studied. Fig.4.15 shows that the cAMP analogue, dibutyryl cAMP, caused a modest stimulation of inhibin-α mRNA levels at a concentration of 100μM. This stimulation was augmented by oestradiol, and to a lesser extent DHT, in a dose-dependent fashion. The only experiment in which the effect of cAMP on expression of inhibin-β_A mRNA was studied (Fig.4.15B) was part of the same experiment presented in Fig.4.14A. In this experiment, basal levels of this message were high, and treatment with dibutyryl cAMP appeared to cause a reduction in inhibin-β_A mRNA levels. However, DHT stimulated

Fig.4.13. Slot-blot analysis of dose-dependent stimulation of inhibin α-subunit mRNA expression by oestradiol in cultured rat granulosa cells. Panel A: duplicate experiments to show the effect of oestradiol in the absence of FSH; 1) control, 2) 10^{-8}M oestradiol, and 3) 10^{-6}M oestradiol. Panel B: triplicate experiments to show the effect of oestradiol in the presence of 30ng/ml FSH; 1) control, 2) 30ng/ml FSH alone, 3) 30ng/ml FSH plus 10^{-8}M oestradiol, and 4) 30ng/ml FSH plus 10^{-6}M oestradiol. Panel C: the effect of oestradiol in the presence of 100ng/ml FSH; 1) control, 2) 100ng/ml FSH alone, 3) 100ng/ml FSH plus 10^{-8}M oestradiol, and 3) 100ng/ml FSH plus 10^{-6}M oestradiol. Cells were cultured for 48h with hormonal treatments before RNA was extracted, and applied to Zeta-Probe membrane using a slot-blot apparatus. Amounts of RNA applied to each slot were 2μg (panel A, experiment [a] and panel B, experiments [a] and [b]) or 8μg (panel A, experiment [b], panel B, experiment [c] and panel C). Slot-blots were probed with a ^{32}P-labelled inhibin α-subunit cDNA. Autoradiograms were exposed for 7 days in all cases except panel B, experiment (c), which was exposed for 18h.

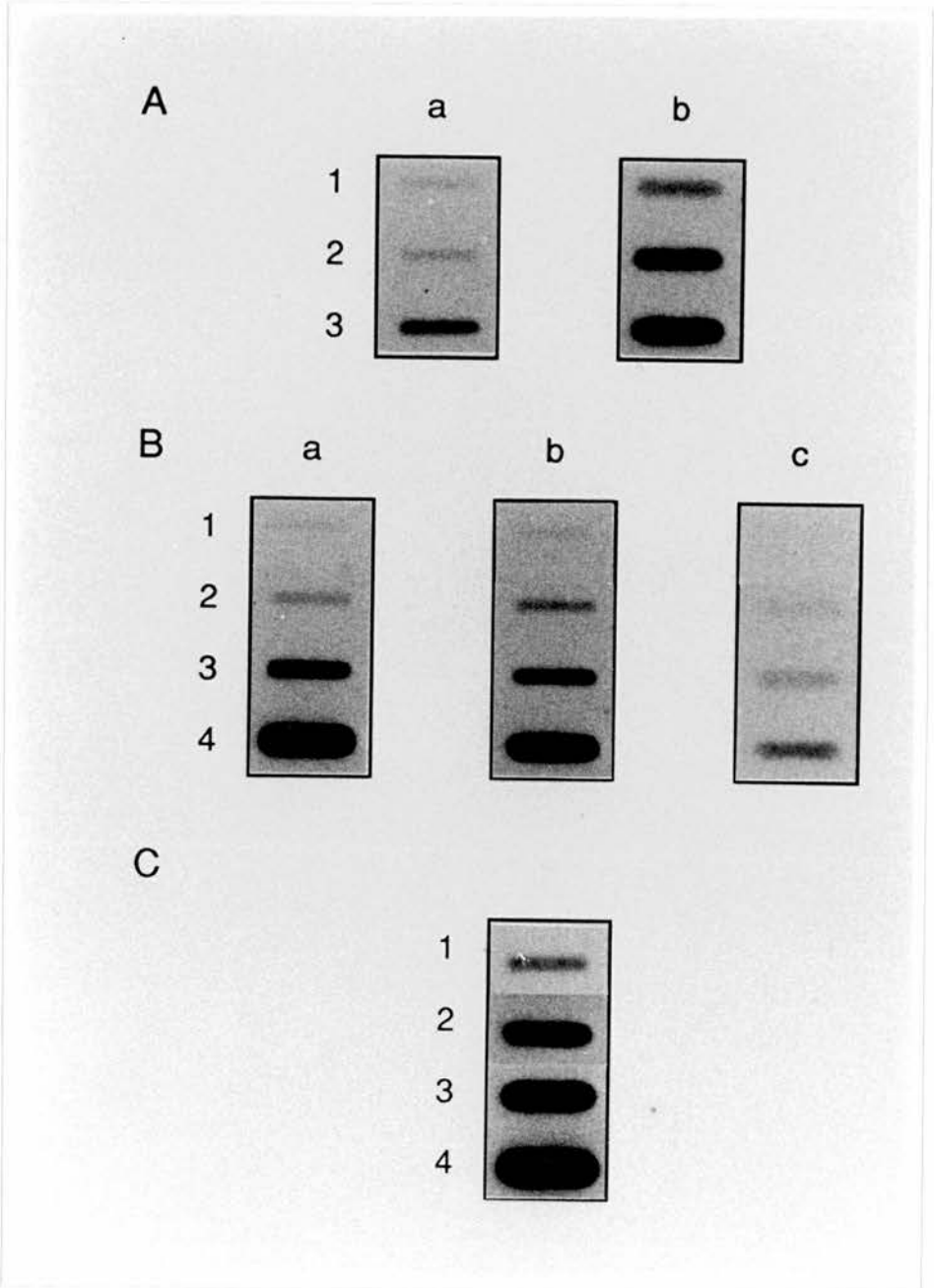

A
a
b
1
2
3
B
a
b
c
1
2
3
4
C
1
2
3
4

Fig.4.14. Dose-dependent effect of DHT on expression of inhibin α- and β_A-subunit mRNA. Rat granulosa cells were cultured for 48h under the following conditions: 1) control, 2) 10^{-8}M DHT, 3) 10^{-6}M DHT, 4) 30ng/ml FSH, 5) 30ng/ml FSH plus 10^{-8}M DHT, and 6) 30ng/ml FSH plus 10^{-6}M DHT. Total RNA was extracted from monolayers, and analysed for inhibin subunit mRNA. Panel A: total RNA (10μg) was subjected to electrophoresis in an agarose-formaldehyde gel, transferred to a nylon membrane, and inhibin α- and β_A-subunit mRNA were detected by Northern blot analysis by hybridisation with ^{32}P-labelled cDNA probes as marked. Autoradiographs were exposed for 24h (α) and 14 days (β_A). The 18s ribosomal RNA bands, stained with ethidium bromide, from the gel used to generate the blot are shown at the bottom to demonstrate even loading of RNA. Panel B: inhibin α-subunit mRNA was measured in total RNA (2μg/slot) from a separate experiment by slot-blot analysis. Exposure was for 7 days.

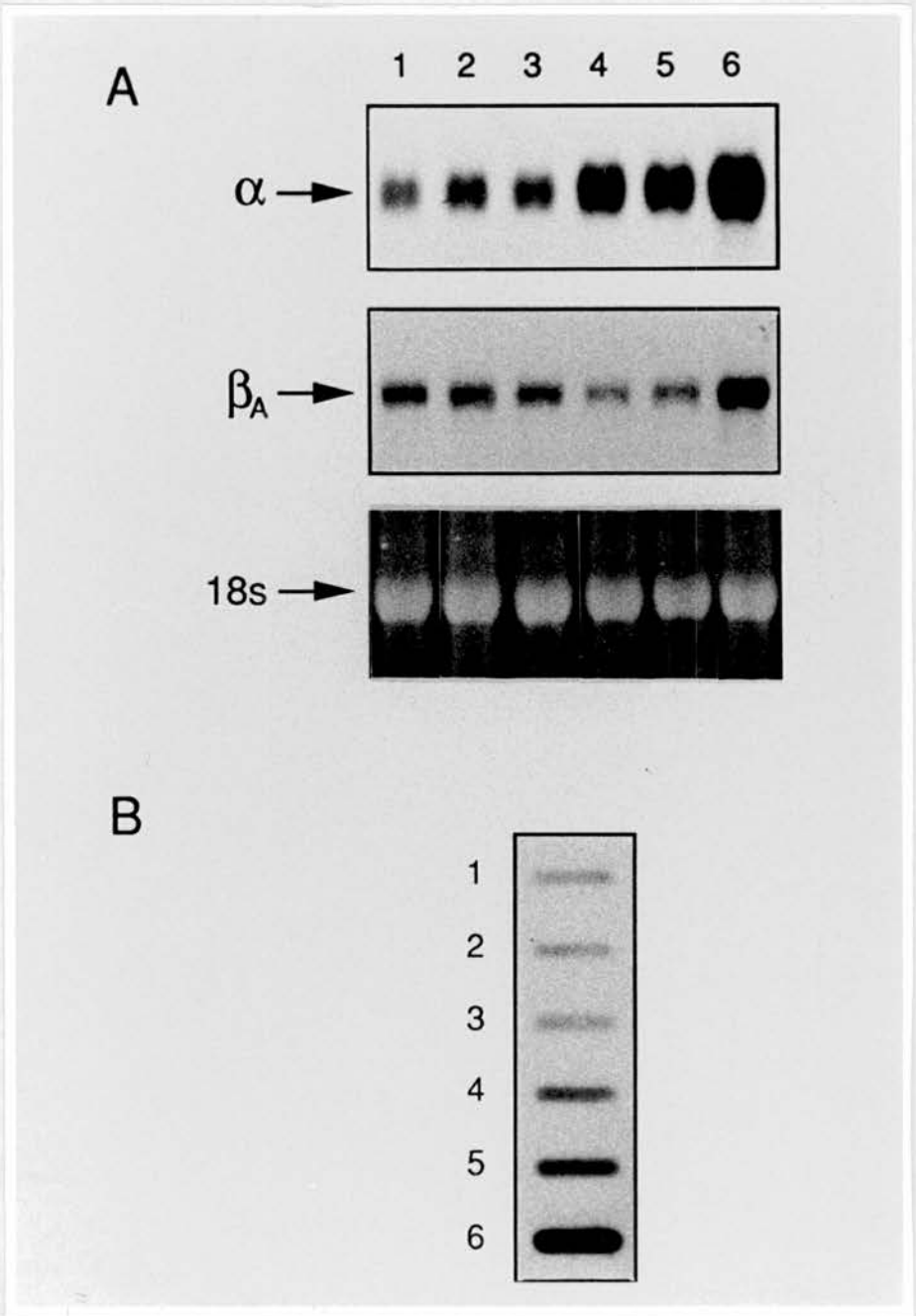
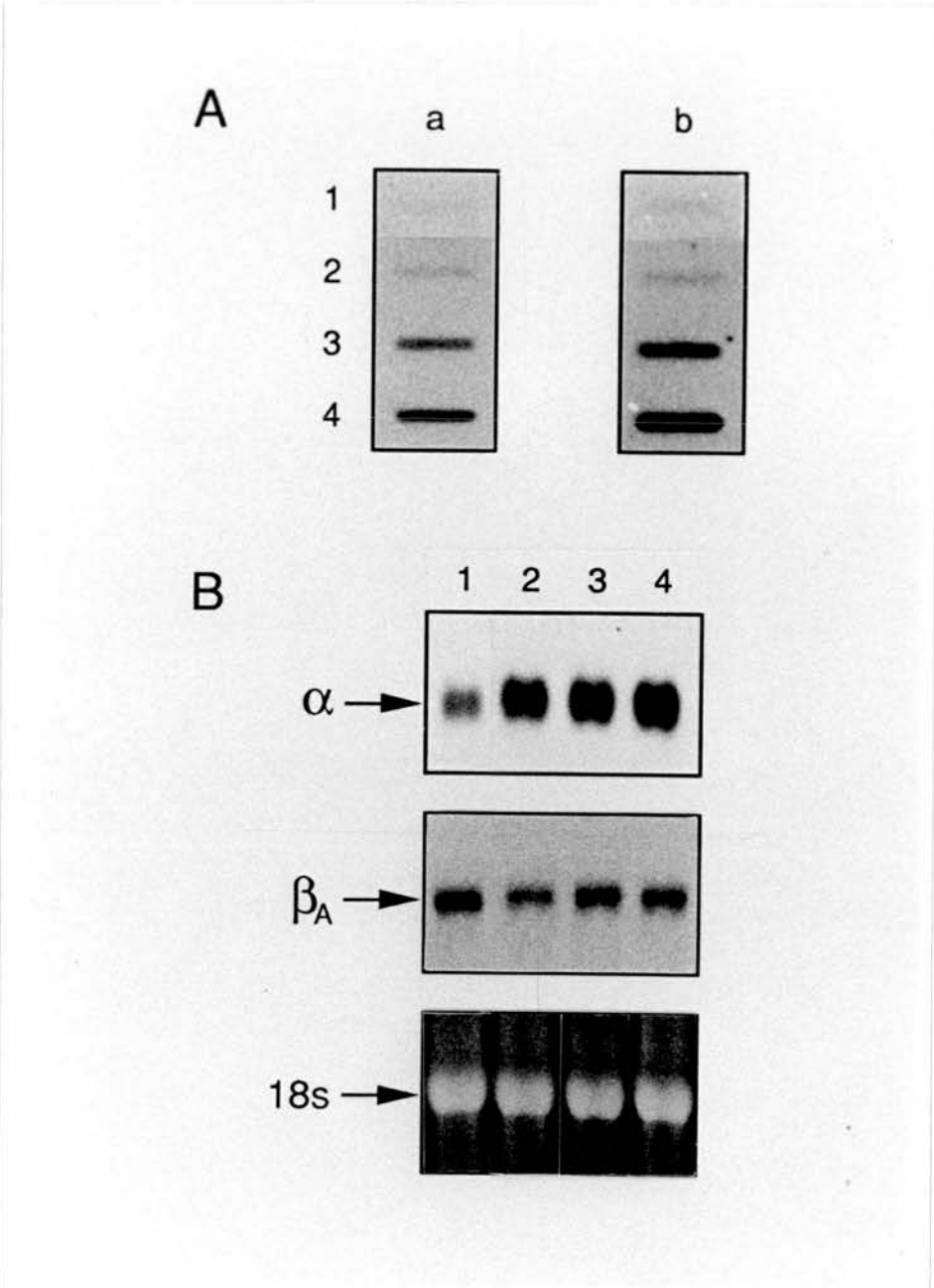

A
1 2 3 4 5 6
α
β_A
18s
B
1
2
3
4
5
6

Fig.4.15. Effects of steroids on cAMP-stimulated inhibin subunit mRNA expression in cultured rat granulosa cells. Panel A: duplicate experiments to show inhibin α-subunit mRNA in granulosa cells cultured for 48h under the following conditions; 1) control, 2) 100μM dibutyryl cAMP, 3) 100μM dibutyryl cAMP plus 10^{-8}M oestradiol, and 4) 100μM dibutyryl cAMP plus 10^{-6}M oestradiol. Total RNA was extracted from cells, and applied to Zeta-Probe membrane using a slot-blot apparatus (1μg [experiment a] or 2μg [experiment b] RNA per slot), and the membrane was hybridised with a rat inhibin α-subunit cDNA probe. Autoradiograms were exposed for 7 days. Panel B: cells were cultured for 48h under the following conditions; 1) control, 2) 100μM dibutyryl cAMP, 3) 100μM dibutyryl cAMP plus 10^{-8}M DHT, and 4) 100μM dibutyryl cAMP plus 10^{-6}M DHT. RNA was extracted and subjected to electrophoresis (10μg/lane) in an agarose-formaldehyde gel, and transferred to a nylon membrane, which was sequentially hybridised with ^{32}P-labelled rat inhibin α- and β_A-subunit cDNA probes, as marked. Autoradiograms were exposed for 24h (α) and 14 days (β_A). The 18s ribosomal RNA bands, stained with ethidium bromide, from the gel used to generate the blot are shown at the bottom to demonstrate even loading of RNA.


A
a
b
1
2
3
4
B
1 2 3 4
α
βA
18s

inhibin-β_A mRNA levels slightly in the presence of cAMP, when compared to cAMP alone.

In order to determine how these effects of gonadotrophins and steroids on inhibin gene expression are reflected in changes in secretion of inhibin protein, the culture medium from some of the experiments described above was assayed for inhibin, using a radioimmunoassay specific for the inhibin α-subunit. The results are shown in Fig.4.16. FSH (100ng/ml) stimulated inhibin secretion. The effect of oestradiol alone on inhibin α- and β_B-subunit gene expression was reflected in an increase in secretion of immunoreactive inhibin, whereas DHT had no effect. The stimulation of immunoreactive inhibin secretion by FSH was augmented by oestradiol and DHT. As with the effects of steroids on levels of inhibin subunit mRNA, oestradiol was more potent than DHT in this regard.

4 Discussion

The results presented in this chapter provide strong evidence that the expression of mRNA encoding the inhibin subunits in rat granulosa cells is under direct control by gonadotrophins *in vivo*. Stimulation of inhibin α-subunit mRNA expression by FSH was confirmed, and a much stronger effect of FSH on the expression of the β-subunits was also demonstrated. It was shown that, after treatment of animals for 48h with FSH, hCG caused a decrease in the levels of mRNA encoding all three inhibin subunits in granulosa cells. This is consistent with a recent report that showed that PMSG, which has both FSH and LH activity, has a biphasic effect on inhibin α- and β_A-subunit mRNA, being stimulatory in the short term and at low concentration, and inhibitory in the longer term and at high doses (Michel *et al.* 1991a). The effects of gonadotrophins on levels of β-subunit mRNA were more pronounced than their effects on α-subunit mRNA, suggesting that the regulation of inhibin synthesis may be exerted mainly at the level of expression of the β-subunits.

The regulation by gonadotrophins of the expression of mRNA encoding P450arom and LH receptor in these cells was found to follow a very similar pattern, being stimulated by FSH, and inhibited by hCG after

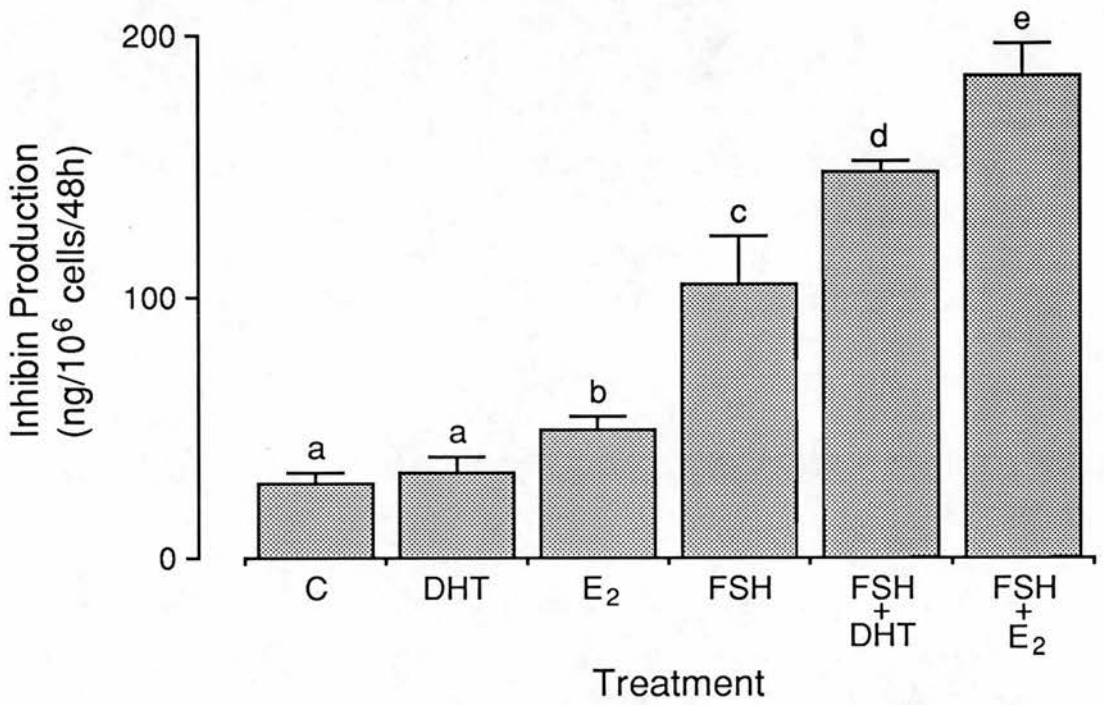


Fig.4.16. Effects of FSH and steroids on secretion of immunoreactive inhibin by rat granulosa cells *in vitro*. Cells from DES-treated animals were cultured in the absence of hormones (C), or in the presence of 10^{-6}M 5α-dihydrotestosterone alone (DHT), 10^{-6}M oestradiol-17β alone (E_2), 100ng/ml hFSH alone (FSH), 100ng/ml hFSH plus 10^{-6}M 5α-dihydrotestosterone (FSH+DHT) or 100ng/ml hFSH plus 10^{-6}M oestradiol-17β (FSH+E_2). Histograms with different superscripts are significantly different from one another ($p<0.05$).

treatment with FSH, suggesting that these genes are regulated in a very similar fashion. It was also found that the mRNA encoding the inhibin subunits, P450arom and the LH receptor could also be stimulated by hCG in the granulosa cells of immature DES-primed rats which received no other treatment. The effects of FSH and hCG are consistent with the results of other studies of the hormonal regulation of levels of mRNA encoding the LH receptor (Peng *et al.* 1991; Segaloff *et al.* 1990) and P450arom (Hickey *et al.* 1988; Fitzpatrick & Richards, 1991). The effect of hCG on the inhibin subunits was consistent, whereas its effect on LH receptor and P450arom mRNA was observed only in one of three experiments. According to the principles of the rat granulosa cell model used throughout this thesis, the granulosa cells of these animals are in an undifferentiated state, and should not, therefore, express receptors for LH and hCG. The effect of hCG observed here, however, casts some doubt on the homogeneity of the state of maturity of granulosa cells obtained by this method. It was demonstrated that expression of LH receptor mRNA was high in the residual ovarian tissue of untreated animals, and was found to be profoundly suppressed by injection of hCG, in agreement with previously published reports (Peng *et al.* 1991). The source of this LH receptor mRNA was probably thecal and interstitial tissue. Therefore, it is possible that the effect of hCG on the granulosa cells of untreated animals, where observed, was mediated via a product of the theca whose production was stimulated by hCG treatment. However, since this effect was variable, it is also possible that the granulosa cells in which hCG increased levels of LH receptor and P450arom mRNA were more mature than those on which hCG had no effect, and already had some degree of LH responsiveness. No LH receptor mRNA was detected in untreated granulosa cells, but this does not necessarily mean that they had no LH receptors, since it has been shown that, although synthesis of LH receptors is closely linked to expression of the mRNA (LaPolt *et al.* 1990a; Segaloff *et al.* 1990), substantial changes in LH receptor synthesis can take place without any change in levels of the mRNA (Wang *et al.* 1991).

The expression of P450scc mRNA followed a different pattern, consistent with previous reports (Goldring *et al.* 1987). As was the case for the other markers of granulosa cell maturity studied, FSH stimulated

levels of P450scc mRNA. However, unlike the levels of mRNA encoding the inhibin subunits, P450arom and the LH receptor, which fell upon hCG treatment in granulosa cells of animals which had received 48h of FSH treatment, P450scc mRNA levels were further stimulated by hCG.

These data are consistent with the present concepts of follicular development, which hold that stimulation of growth of follicles by FSH is associated with increased capacity of the granulosa cells to produce oestradiol and to respond to LH. Evidently, increased capacity to produce inhibin and/or activin is also intimately associated with the growth of the follicle. As discussed in Chapter 1, this secretion of oestradiol and inhibin is thought to lead to suppression of FSH secretion by the pituitary. The increased responsiveness of the granulosa cells of highly developed follicles to LH allows such follicles to continue growing in the face of falling FSH levels. In the rat, following the ovulatory LH surge, secretion of inhibin and oestradiol fall (Butcher *et al.* 1974; Hasegawa *et al.* 1989; Watanabe *et al.* 1990), allowing FSH levels to rise, leading to recruitment of another cohort of primary follicles, while progesterone secretion by the newly formed corpus luteum rises.

Because of the potent effects of steroids in modulating FSH action (see Chapters 1 and 2), the possible effects of steroids in modulating the effect of FSH on the expression of the inhibin subunits genes were examined using the granulosa cell culture system. Initially, the changes in expression of the inhibin subunit genes in response to gonadotrophins *in vitro* were studied in the absence of steroids, and were found to be similar to those observed *in vivo*. FSH caused an increase in levels of mRNA encoding all three inhibin subunits, an effect which was strongly reversed by subsequent treatment with hCG. Again, LH receptor and P450arom mRNA followed the same pattern as the inhibin subunits, whereas P450scc message levels were stimulated by FSH, and further stimulated by hCG after FSH treatment.

The most obvious differences between the expression of the inhibin subunits *in vitro* and *in vivo* were a much lower basal level of inhibin α-subunit mRNA in cultured cells, and an absence of effect of hCG on expression of inhibin subunit mRNA in cells which had not been treated with FSH. It is possible that the cells used for the *in vitro* experiments

were less mature than those used for the *in vivo* experiments. However, the stimulation of inhibin subunit mRNA by hCG *in vivo*, and the ineffectiveness of hCG *in vitro* were consistently observed, suggesting that the effect of hCG was indeed mediated via (a) theca cell product(s). It is possible that granulosa cells lose their responsiveness to hCG when placed in culture, but they do remain responsive to FSH, and acquire LH responsiveness in response to FSH *in vitro*. The lower levels of inhibin-α mRNA in untreated cultured granulosa cells, when compared to levels in freshly isolated cells, also suggest that there is some factor present *in vivo*, driving inhibin-α mRNA expression, which is absent *in vitro*.

The effect of FSH alone *in vitro* was rather variable, further pointing to the variable degree of maturity of cells isolated from immature rats treated with DES. The modulatory effect of steroids appeared to be heavily dependent upon the degree of stimulation brought about by FSH alone. In experiments in which FSH had a strong stimulatory effect on inhibin subunit mRNA expression, steroids appeared to have no further effect. However, where the effect of FSH was small, strong evidence for augmentation of the effect of FSH by both androgen and oestrogen was found. Steroids also were found to augment the effect of cAMP, consistent with cAMP being the major second messenger mediating the effects of FSH.

These results were corroborated by the secretion of inhibin into the medium, as measured by RIA. It should be noted that the assay measures free inhibin-α as well as mature inhibin (Sharpe *et al.* 1988). It has been shown that granulosa cells of several species secrete free α-subunit molecules (Bicsak *et al.* 1988; Knight *et al.* 1989; Schneyer *et al.* 1990), which is consistent with the apparently higher levels of mRNA encoding the α-subunit than the β-subunit under control conditions (judging by the exposure times required for detection by Northern blotting).

All these results are consistent with current knowledge of steroid action in granulosa cells, and of the changes in hormone secretion during the oestrous cycle. Interestingly, it was found that oestradiol was more potent than DHT in augmenting the effect of FSH on inhibin subunit gene expression, as well as on immunoreactive inhibin secretion. This is surprising because other steroid-responsive end-points studied to date in

granulosa cells are generally more sensitive to androgen than oestrogen (Armstrong & Dorrington, 1976; Hillier *et al.* 1985; Hudson *et al.* 1987; Wang & Leung, 1987 a). Even more surprising was the finding that oestradiol stimulates expression of mRNA encoding the inhibin α- and β_B-subunits in the absence of FSH. This is difficult to reconcile with the present understanding of the role of steroids in the control of granulosa cell function, since it is thought that androgens and oestrogens act by amplifying the elevation of intracellular cAMP levels stimulated by FSH, but not by stimulating cAMP generation independently of FSH. It could be that there is a very small basal level of cAMP generation in these cells, which is enhanced by oestradiol. If expression of the inhibin subunit genes were particularly sensitive to stimulation by cAMP, this mechanism could be sufficient to explain the effect of oestradiol in the absence of FSH. However, this does not explain why DHT does not have the same effect alone, or why oestradiol appears to be more potent than DHT in augmenting FSH-stimulated inhibin gene expression.

It is difficult to envisage a situation where there is sufficient P450arom present in granulosa cells to produce stimulatory concentrations of oestradiol in the complete absence of FSH. However, differential control of inhibin subunit expression by FSH and oestradiol may influence the forms of inhibin or activin which are produced in different hormonal environments. It has been shown that oestrogen may inhibit thecal androgen production (Leung & Armstrong, 1979; Magoffin & Erickson, 1982), while inhibin stimulates androgen synthesis (Hsueh *et al.* 1987; Hillier *et al.* 1991b). Therefore, the stimulation of inhibin production by oestrogen could constitute a positive feedback mechanism whereby oestrogen maintains the supply of thecal androgen to act as a substrate for oestrogen synthesis despite high local levels of oestrogen.

Although these results provide strong evidence for stimulation of inhibin subunit gene expression by FSH and by oestradiol, and augmentation of the action of FSH by sex steroids, several questions remained unanswered. Firstly, a knowledge of the relative amounts of inhibin α-, β_A- and β_B-subunit mRNA species expressed under different stimuli would be useful for the prediction of the forms of inhibin or

activin which are produced by granulosa cells under those circumstances, and for the understanding of the molecular biology of inhibin and activin synthesis. Secondly, the three genes appeared to be regulated by FSH in a similar fashion to one another, but differentially regulated by oestradiol alone. However, due to the apparently lower abundance of inhibin-β_A mRNA, it is possible that there is a direct effect of oestradiol on the expression of this message, which is not detectable by the method used here. The third, and most important, remaining question about the regulation of inhibin subunit gene expression concerns the stage or stages of granulosa cell differentiation at which inhibin and activin are produced. A number of potential ovarian paracrine and autocrine feedback mechanisms have been proposed, by which the development of the follicle is controlled (Adashi *et al.* 1985a; Hsueh, 1986; Ledwitz Rigby, 1987; Terranova, 1991; Hillier, 1991; Hsueh *et al.* 1984). Central to these mechanisms is the action of FSH in inducing various genes in granulosa cells, but since the sequence of induction of different genes is not yet clear, it is not yet known which potential feedback mechanisms operate at what stage of follicular development. One hypothesis to explain the regulation by gonadotrophins of expression of different genes in the ovary at different times during the cycle is based upon differential sensitivity of genes to stimulation by cAMP (Richards *et al.* 1987; Yong *et al.* 1991a). Therefore, the sensitivity of important granulosa cell genes to stimulation by cAMP and steroids could give valuable insights into the sequence of events taking place during the differentiation of granulosa cells. Based on the data presented here, it could be hypothesised that the inhibin subunit genes have a lower threshold for expression than P450arom, suggesting that inhibin and/or activin may be important in local control of the early stages of follicle development.

Extensive dose-response experiments would be necessary to test this hypothesis. Unfortunately, the techniques used in the experiments described in this chapter are not adequate for the analysis of complex experiments. Northern blotting requires large numbers of cells to provide sufficient quantities of RNA for analysis, and only a limited number of samples can be analysed on the same membrane. Also, Northern analysis provides only semi-quantitative data, such that direct comparison cannot

be made between autoradiographic signals obtained from different membranes. Indeed, considerable variation was found when making comparisons of intensities of signals obtained from different exposures of the same blot. Therefore, in order to study quantitatively the dose-response characteristics of the regulation of the inhibin subunit genes, and to determine the relative amounts of the different gene products, a more sensitive and reproducible assay was required. The method of choice was a solution hybridisation RNase protection assay.

Chapter 5 Development of an RNase Protection Assay for Inhibin-α

1 Introduction

Data obtained by Northern blotting, and presented in the previous chapter, clearly showed that FSH stimulates the expression of all three inhibin genes in rat granulosa cells, and that steroids are able to modulate this effect of FSH. However, as discussed in the conclusion of that chapter, in order to fully understand the regulation of these genes, a considerable amount of quantitative data are still required; firstly, to measure the absolute levels of each mRNA species, which would aid the understanding of the synthesis of forms of inhibin and activin; secondly, to investigate the dose-response characteristics of stimulation of inhibin gene expression by steroids, FSH and the presumptive second messenger, cAMP, in order to understand the timing of production of inhibin and activin during the development of the follicle.

The technique of Northern analysis, which is almost universally used to measure levels of specific mRNA species, is based on hybridisation of labelled probes with RNA immobilised on membranes, followed by detection of hybridisation by autoradiography. Originally these methods were developed by molecular biologists for the purpose of detecting qualitative differences in gene expression between samples (for example, in identifying a tissue or experimental situation in which a particular gene is being expressed, when compared to one in which it is not), and there are advantages to the use of this approach. Since RNA detected by Northern analysis has been separated by electrophoresis, the molecular weights of specific mRNA species can be calculated. This method also reveals the number of transcripts which hybridize to the probe. This allows differentiation between functional mRNA molecules, related or incomplete transcripts, and background hybridization. The hybridization of a probe to RNA on a membrane is determined by the

stringency of the hybridization and washing, and the degree of sequence homology (i.e. the number of matching nucleotides) between the probe and the RNA. As a result, a signal detected by Northern analysis may not share complete sequence identity with the probe. This allows use of a cDNA from one species to detect expression of the corresponding mRNA in a different species, or to screen a cDNA library for a related sequence. Also, the process of generating a Northern blot is straightforward and, once the RNA is fixed to the nylon, the same blot may easily be probed, stripped and reprobed for several different mRNA species.

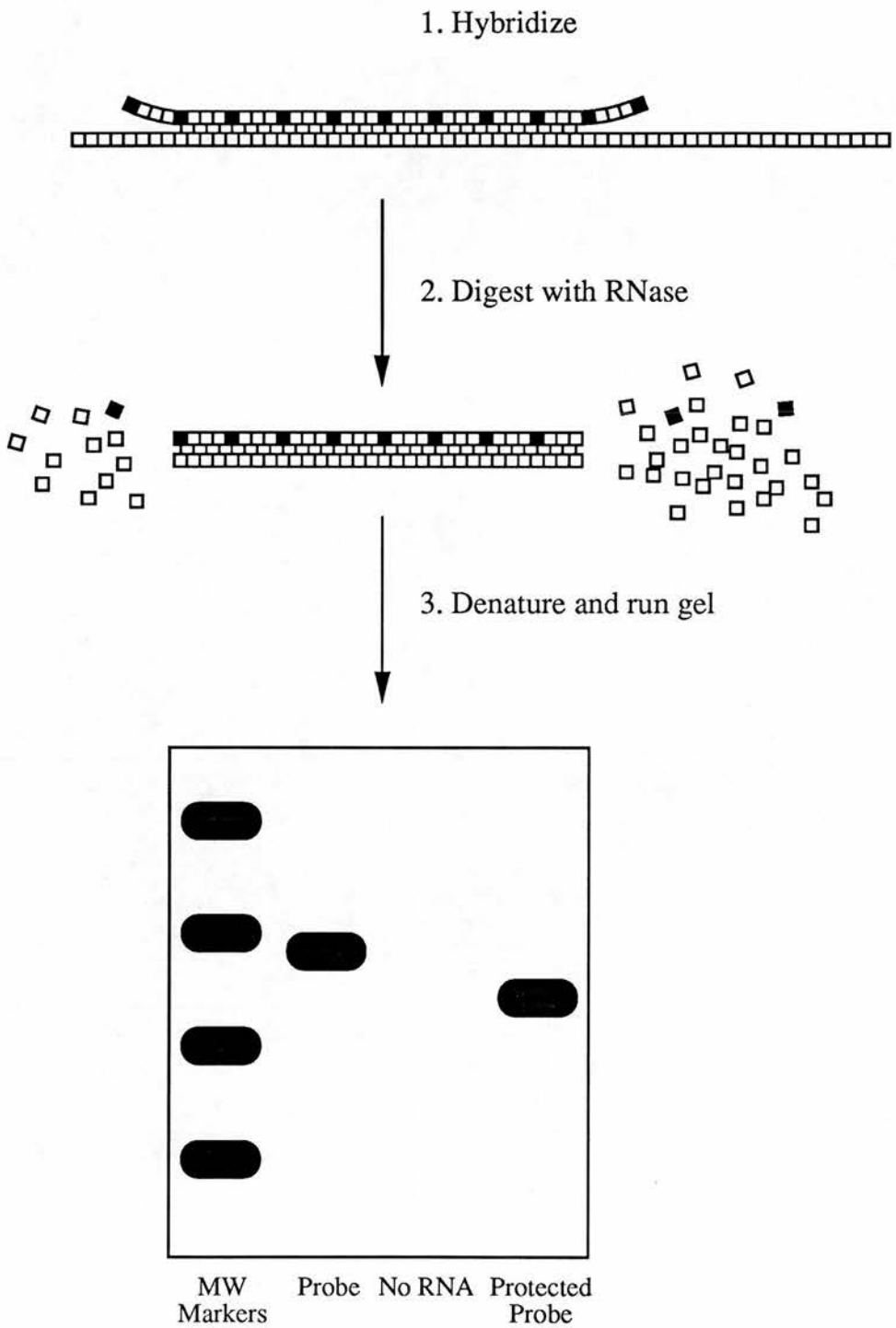
However, this technique is not suitable for quantitative studies of gene expression. Efforts have often been made to derive quantitative information from autoradiographs by measuring the optical densities of signals using densitometry. However, in addition to the abundance of a specific mRNA, there are several other variables influencing the strength of an autoradiographic signal from a Northern blot; the efficiency and evenness of transfer of RNA from the gel to the membrane, the degree of fixation of RNA to the membrane, the concentration and specific activity of the probe, the uniformity of hybridization and washing of the probe across the membrane, the use of intensifying screens and the length of exposure of the blot to the film all can influence signal strength. Also, the relationship between optical density and radioactive exposure is non-linear at both low and high signal intensities (Swillens *et al.* 1989). Therefore, if the intensities of two signals are to be compared, both must fall within a range over which this relationship is linear. Since it is not known before the development of the film whether the signals obtained will all fall within this range, the optimum exposure time is difficult to acheive. The best degree of quantitation that can be achieved in this way is a comparison of arbitrary densitometric units between spots on the same autoradiogram. Relative abundances of different transcripts in an RNA sample immobilised on a membrane cannot be measured by densitometry, since detection of different mRNA species may require different exposure times.

Another disadvantage of Northern analysis is the amount of RNA the method requires. Abundant mRNA species such as inhibin α-subunit mRNA were detected by Northern analysis of less than 5μg of total

granulosa cell RNA after exposure of blots for 1-2 days (see previous chapter, Figs.4.10-4.12). However, in order to detect relatively rare transcripts, such as aromatase message, more RNA had to be analysed and exposure times extended. The typical yield of total RNA from cultured cells has been approximately 4μg per million cells. For studies on the expression of inhibin subunit genes, the number of cells needed for each treatment was 3-4million cells, which would yield enough RNA for two or three Northern blots. As two ovaries will typically yield approximately 1-2 million cells, cells from at least two animals were needed for each treatment in a culture. As a result, the design of the experiments was limited by the logistics of setting up very large-scale cultures, and the availability of animals.

Because of these limitations of Northern analysis, a more quantitative and sensitive detection method was required to study the regulation of inhibin subunit gene expression in detail. The most direct technique for the quantitative measurement of specific mRNAs is by solution hybridization RNase protection assay. This method is based upon the the fact that perfectly matched RNA:RNA hybrids are highly resistant to digestion with RNases A and T_1, whereas unhybridized (single-stranded) RNA is extremely susceptible to degradation by those enzymes. The method is summarized in Fig.5.1. A uniformly labelled complementary RNA probe is incubated in solution with a sample of total cellular RNA containing an unknown quantity of the target mRNA and hybridization is allowed to take place. The sample is then incubated with a mixture of RNases A and T_1, which degrades all RNA not present as perfectly matched hybrids. If the probe is present in an excess over the target sequence, the amount of probe protected from RNase digestion is proportional to the amount of target mRNA present in the original sample. The amount of probe protected can be measured by visualization on a polyacrylamide gel, or by scintillation counting of protected probe adsorbed to charged paper filters. Two controls are included, containing the same amount of probe as the samples, but no RNA; a background control, in which the probe is digested with RNase, and an input control, in which it is not.

Fig.5.1. Diagrammatic representation of the RNase protection assay technique. 1. A uniformly labelled cRNA probe (represented by the upper strand, black squares denoting radioactive nucleotides) is allowed to hybridize to the mRNA of interest (lower strand). 2. RNase is added, digesting all RNA not forming perfectly matched hybrids. 3. RNase-resistant fragments are visualized by electrophoresis and autoradiography.


1. Hybridize
2. Digest with RNase
3. Denature and run gel
MW Markers
Probe
No RNA
Protected Probe

Because of its ability to detect sequence mismatches with extreme accuracy, the major applications of this technique have been in the mapping of intron/exon junctions in primary gene transcripts (Hernandez *et al.* 1989; White *et al.* 1987) and in the detection of point mutations and deletions (Garcia *et al.* 1988). However, it is also a useful method for the measurement of the abundance of specific mRNAs (Werner *et al.* 1989; Leung *et al.* 1987; Segaloff *et al.* 1990). The advantages of this method of mRNA analysis over Northern blotting are greater sensitivity and quantitative measurement of mRNA abundance. It has been suggested that RNase protection assay is at least tenfold more sensitive than Northern analysis, being able to detect as little as 0.1pg of target mRNA (Melton *et al.* 1984). The greater sensitivity of this method is due to a greater extent of hybridisation that can be achieved. This is a result of the low hybridisation volume used, compared to membrane hybridisation methods, allowing much greater probe concentrations to be used (Hames & Higgins, 1987). Also, RNA in solution is more accessible to hybridisation than RNA immobilised on a membrane (Sambrook *et al.* 1989). The problems associated with autoradiography as a detection method can be circumvented by measuring the amount of probe protected using liquid scintillation counting. Synthetic sense RNA can be used to construct a standard curve of known concentration, and quality control samples can be included in assays, so that quantitative and reproducible data can be obtained. Because less RNA is required for analysis by this method, samples can be assayed in duplicate or triplicate, allowing statistical analysis to be performed.

Probes for analysis of mRNA by RNase protection assay must be single-stranded, uniformly labelled antisense RNA. In addition, unlabelled sense RNA is required to act as a standard. RNA probes can be prepared by transcription *in vitro*, using the appropriate strand of the cDNA as a template. In order to do this, the cDNA must be inserted into a plasmid carrying sequences on either side of the cDNA which act as promoter elements for commercially available bacteriophage DNA-dependent RNA polymerases, allowing transcription of RNA from either strand. Since pUC18, the plasmid into which the inhibin-α cDNA was supplied, bears no such promoter sequences, it was necessary to

subclone the cDNA into a suitable vector. The vector which was chosen was pGEM3Zf(+) (Promega) (Fig.5.2). This plasmid contains sequences on either side of the multiple cloning site which act as promoters for bacteriophage SP6 and T7 RNA polymerases, which can therefore be used to generate RNA transcripts from either strand.

The aims of this section of the project were to subclone the inhibin α-subunit cDNA into pGEM3Zf(+), to use synthetic RNA derived from this construct to develop and validate a sensitive and reproducible RNase protection assay for the quantitative measurement of mRNA encoding the inhibin α-subunit, and to use this assay to study in detail the hormonal regulation of inhibin-α gene expression.

2 Materials and Methods

Animals, *in vivo* hormonal treatments, isolation of granulosa cells, extraction of RNA, and Northern analysis were all as described in section 2.2. The following methods were also used in the studies described in this chapter.

2.1 Subcloning of Inhibin α-Subunit cDNA

The α-subunit clone (α7; see Fig.5.3) contains in its 5' end sequences not confirmed by the other α-subunit clone obtained by the group supplying the clone. This region contains multiple termination codons with a putative amino acid coding region (Esch *et al.* 1987). Therefore, the *Hinf*I/*Kpn*I fragment of the cDNA (1027bp long) was chosen for subcloning. This fragment contains most of the open reading frame and none of the untranslated regions of the α-subunit cDNA. The strategy used for the subcloning of this fragment is summarised in Fig.5.4.

Inhibin-α/pUC18 was digested with *Hinf*I and *Kpn*I. The resulting DNA fragments were resolved by electrophoresis in a 0.8% agarose gel, and the desired fragment was cut from the gel and purified as described in chapter 2, sections 2.6.3 and 2.6.4. The ends of the α-subunit *Hinf*I/*Kpn*I fragment were filled in by incubation for 30min at room temperature with 10U Klenow fragment of *E. coli* DNA polymerase I, in a buffer

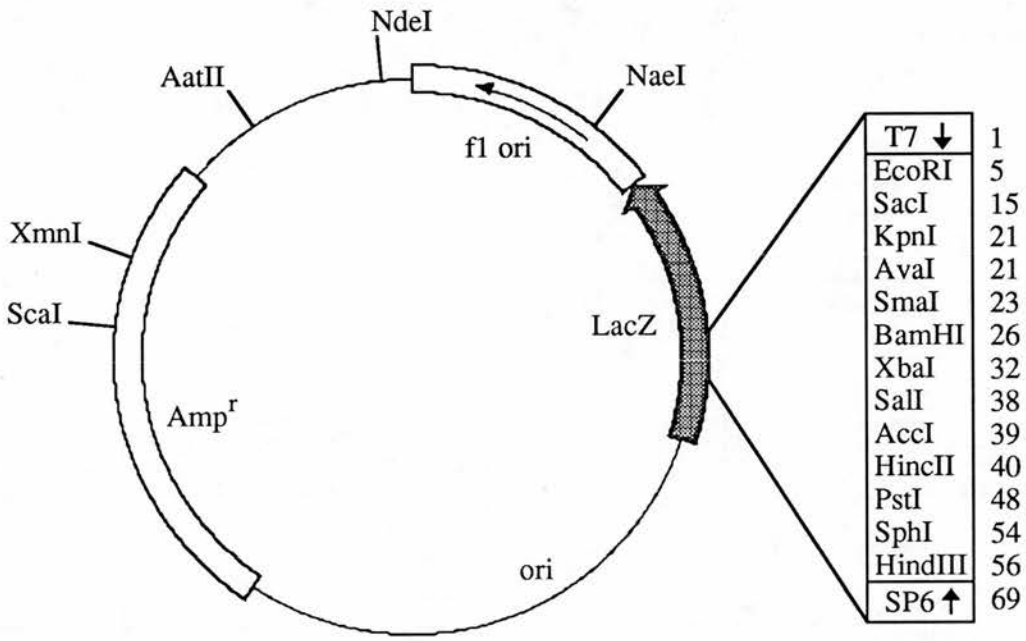


Fig.5.2. Plasmid pGEM3Zf(+) (Promega), showing the LacZ gene, bacteriophage f1 region (f1 ori), bacterial origin of replication (ori) and the ampicillin resistance gene (Amp^r). Unique restriction sites are shown. The multiple cloning site and the flanking promoter regions for bacteriophage T7 and SP6 RNA polymerases are shown on the right. The size of the plasmid is 3199 base pairs.

Fig.5.3. Rat inhibin α-subunit cDNA fragments. A: Diagram of the full length (1336bp) cDNA. The open reading frame is boxed, showing the regions encoding the pro-, αN and mature αC portions of the inhibin α-subunit protein. Non-coding regions are shown as lines. B: Clone α7 (Esch *et al.*, 1987), aligned with the full length cDNA above. The confirmed inhibin-α sequences are boxed, and the unconfirmed 5' region is shown as a line. C: Partial restriction map of the 1027bp *Hinf*I-*Kpn*I fragment of clone α7, aligned with the full length cDNA and clone α7 above. Positions of restriction sites are indicated (in bp) with respect to the 5' end of the full length cDNA. This fragment was subcloned for use as a template for RNA probe generation.

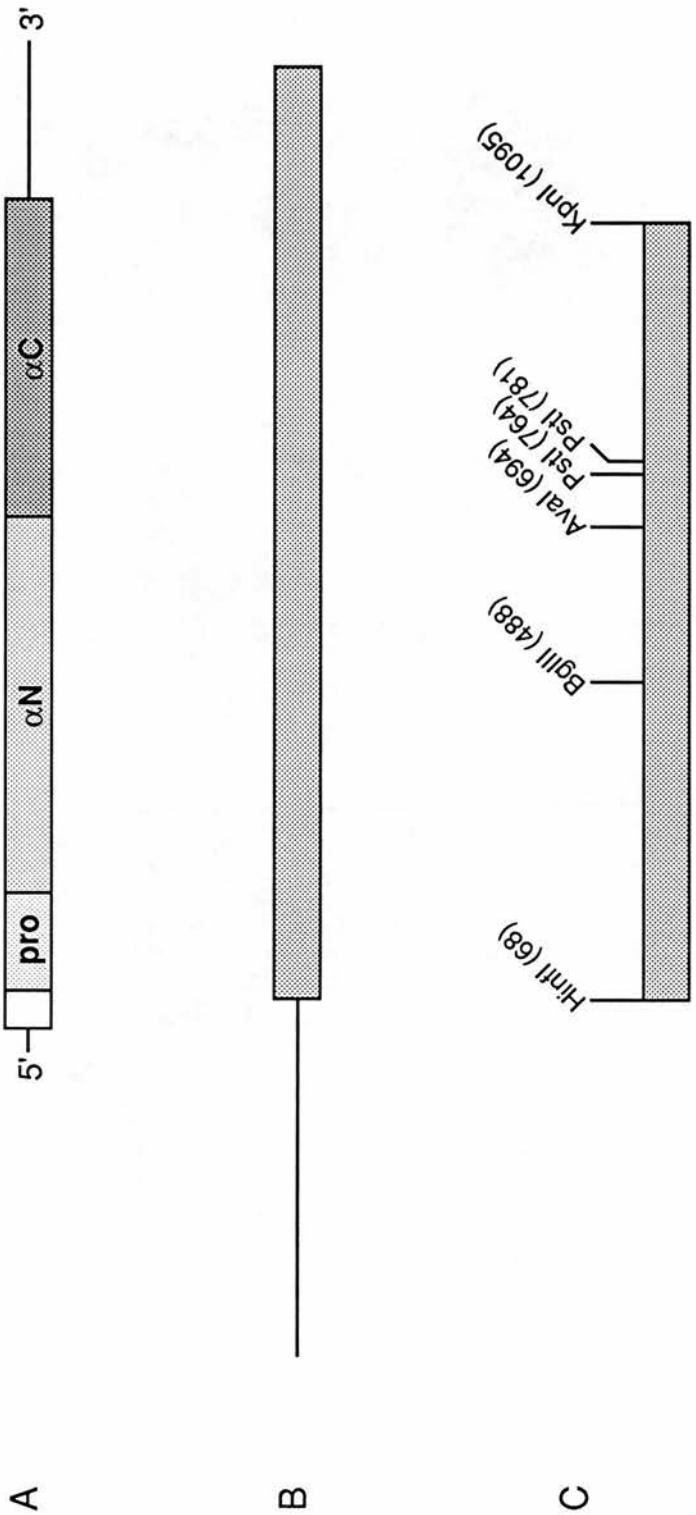
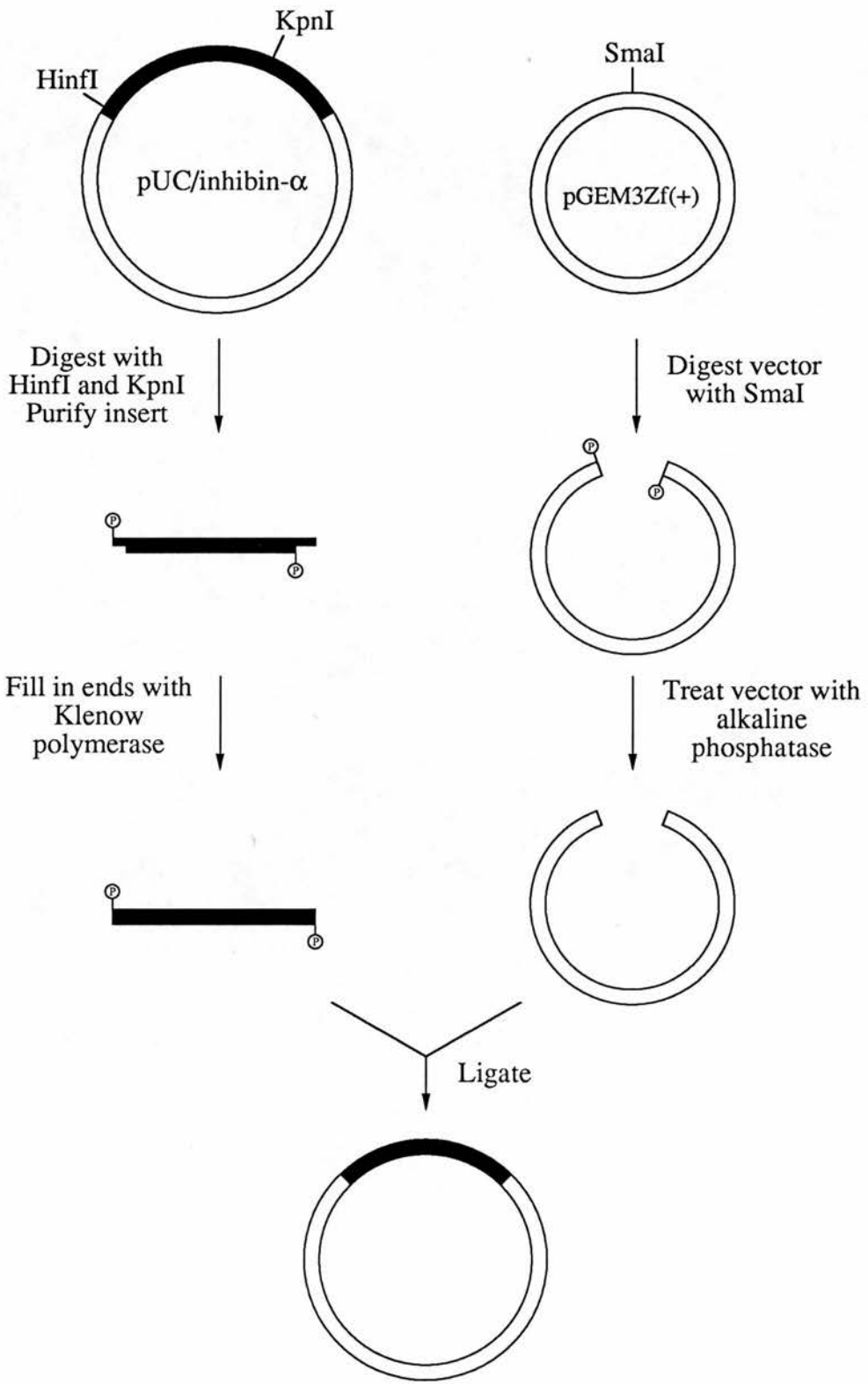

3'
αC
αN
pro
5'
A
B
C
KpnI (1095)
PstI (781)
PstI (764)
AvaI (694)
BglII (488)
HinfI (68)

Fig.5.4. Strategy adopted for subcloning the rat inhibin α-subunit *Hinf*I-*Kpn*I 1027bp cDNA fragment. Parent plasmid (pUC/inhibin-α), containing clone α7, was digested with *Hinf*I and *Kpn*I, and the 1027bp fragment was purified from an agarose gel. The ends of this fragment were then filled in with the Klenow fragment of E.coli DNA polymerase I, to generate blunt ends. The plasmid (pGEM3Zf(+)) into which the cDNA was to be subcloned was digested with *Sma*I, generating blunt ends. Phosphate groups (denoted by the letter P circled) were removed from the linearised plasmid by treatment with calf intestinal alkaline phosphatase, to prevent recircularisation of the plasmid. The blunt-ended inhibin-α 1027bp cDNA fragment was ligated into the *Sma*I site of the plasmid using T4 DNA ligase.


KpnI
HinfI
pUC/inhibin-α
SmaI
pGEM3Zf(+)
Digest with
HinfI and KpnI
Purify insert
Digest vector
with SmaI
Fill in ends with
Klenow
polymerase
Treat vector with
alkaline
phosphatase
Ligate

containing 80μM deoxyribonucleotide triphosphates, 50mM **Tris** (pH7.5), 10mM $MgSO_4$, 100μM DTT and 50μg/ml BSA. The reaction was stopped by the addition of 1μl of 0.5M EDTA (pH8.0), the mixture was extracted once with an equal volume of phenol:chloroform (1:1), and the DNA was purified from unincorporated nucleotides by chromatography on a Sephadex G-50 column (Nick Column, Pharmacia; see chapter 2, section 2.6.4). DNA was recovered by precipitation at -20C for 1h with 0.1 volume of 3M sodium acetate (pH7.0) and 2 volumes of ethanol. In order to accept the blunt-ended α-subunit insert, ~10μg of plasmid were digested with *Sma*I. To prevent recircularization of the plasmid during ligation, 5' phosphate groups were removed from the linearised plasmid by incubation for 1h at 37C with 1U of calf intestinal alkaline phosphatase, in a buffer containing 50mM **Tris** (pH9.0), 1mM $MgCl_2$, 100μM $ZnCl_2$ and 1mM spermidine. The mixture was extracted once with an equal volume of phenol:chloroform, and once with an equal volume of chloroform, and the plasmid DNA was recovered by precipitation at -20C for 1h with 0.1 volume of 3M sodium acetate (pH7.0) and 2 volumes of ethanol.

Blunt-ended inhibin α-subunit 1027bp cDNA fragment was ligated into the *Sma*I-digested, alkaline phosphatase-treated plasmid as follows. 1μg of plasmid and a twofold molar excess of insert (700ng) were incubated with 2 Weiss units of T4 DNA ligase, in a buffer containing 50mM **Tris** (pH7.6), 10mM $MgCl_2$, 1mM ATP, 1mM DTT and 5% (w/v) polyethylene glycol-8000. Ligation reactions were incubated at 4C overnight. Whole ligation reactions were used to transfect competent JM109 cells by the method described in chapter 2, section 2.6.3. Mini plasmid preparations were made from selected clones (see chapter 2, section 2.6.3) and plasmids were digested with *Eco*RI, *Sal*I, and a combination of the two. These enzymes cleave the plasmid on either side of the *Sma*I site, into which the insert was ligated. Therefore, a recombinant plasmid of the expected construction would be linearized by digestion with either enzyme, and the insert liberated by digestion with both. Restriction digests and undigested plasmids were then analysed by Southern blotting. DNA was resolved by electrophoresis in a 0.8% agarose gel (see chapter 2, section 2.6.3). After electrophoresis, the gel was soaked for 45min in denaturing solution (1.5M NaCl, 0.5M NaOH) to separate

the DNA strands, rinsed in distilled water, and then soaked for 45min in two changes of neutralizing solution (1.5M NaCl, 1M Tris pH7.4). DNA was transferred to a nylon membrane (Hybond-N, Amersham) by capillary blotting, using 10xSSC as the transfer buffer, essentially as described for Northern blotting in chapter 2, section 2.6.2. After transfer, DNA was fixed to the membrane by exposure to 254nm UV light for 6min. The blot was probed with a random primed inhibin-α cDNA probe, as described for Northern blot hybridisation in chapter 2, sections 2.6.4 and 2.6.6, to identify positive clones. The orientation of the insert in a positive clone was determined by digestion with *Eco*RI and *Bgl*II. In order to confirm the orientation of the insert, unlabelled RNA was transcribed with SP6 and T7 RNA polymerases using plasmid linearised with *Bgl*II as a template. To act as a sense RNA standard, unlabelled RNA was also transcribed from the entire insert with SP6 RNA polymerase using plasmid linearised with *Eco*RI as a template. Transcription of unlabelled RNA was carried out as described for transcription of ^{32}P-labelled RNA probes (chapter 2, section 2.6.5), except that all ribonucleotide triphosphates were present at a concentration of 500μM, and no labelled nucleotide was present. The sizes of transcripts obtained from each reaction were determined by polyacrylamide gel electrophoresis as described below. The concentration of synthetic sense RNA was determined by spectrophotometry.

In order to demonstrate detection of native inhibin-α mRNA using the presumptive probe, identical Northern blots were probed with ^{32}P-labelled RNA probes transcribed from plasmid linearised with *Bgl*II using SP6 and T7 RNA polymerases, as described in chapter 2, section 2.6.6.

2.2 Polyacrylamide Gel Electrophoresis of RNA

Acrylamide monomer stock solution was obtained as a 40% (w/v) solution of acrylamide and bisacrylamide in a ratio of 19:1 (IBI). Gel stock solution consisted of 4% or 6% acrylamide/bisacrylamide containing 7M urea and 1xTBE. Gels were prepared by the addition of TEMED and APS to the gel stock to a final concentration of 0.08% (v/v and w/v, respectively). Gels were poured between glass plates, using a commercial

polyacrylamide gel apparatus (BioRad), and allowed to polymerize for 1h at room temperature.

Loading buffer (80% [v/v] formamide, 1mM EDTA, 0.1% [w/v] xylene cyanol and 0.1% [w/v] bromophenol blue) was added to 2µl of each sample, which were then heated to 80C for 10min to denature RNA, and placed on ice. An equal volume (10µl) of each sample was then loaded onto adjacent lanes of gels. Electrophoresis was carried out for approximately 45min at 200V.

Unlabelled RNA was visualized by ethidium bromide staining. Gels were stained by soaking for 30min in 1xTBE containing 0.5µg/ml ethidium bromide, and photographed under UV light. Radiolabelled RNA fragments were detected by autoradiography. Gels were fixed by soaking in 10% (v/v) methanol, 10% (v/v) acetic acid for 30min, and then dried onto Whatman 3MM paper, using a commercial gel dryer (BioRad). Dried gels were exposed to Kodak X-Omat AR-5 film overnight at -70C with two intensifying screens.

2.3 RNase Protection Assay

Complementary ^{32}P-labelled RNA probe was transcribed *in vitro* and incorporation of radioactivity and yield of labelled probe were calculated as described in chapter 2, section 2.6.4. Specific activity of probe was 1-2x10^8cpm/µg. Probe was precipitated with 0.1 volume of 3M sodium acetate (pH5.5) and 2.5 volumes of ethanol at -70C for 30min. Probe was redissolved in hybridisation buffer to a final concentration of 10^4cpm/µl. In all cases, hybridisation buffer contained 0.05% (w/v) SDS, 10mM **Tris** (pH7.4), 0.6M NaCl, and 2mM EDTA, after Leung *et al.* (1987). Because RNA may suffer non-enzymatic hydrolysis in solution at high temperatures (Tenhunen, 1989; Hames & Higgins, 1987), many protocols for RNase protection assays include 50-80% formamide in the buffer (Melton *et al.* 1984; Hernandez *et al.* 1991; Lynn *et al.* 1983), so that lower hybridisation temperatures can be used. Therefore, assays were carried out using this buffer without formamide, and also with 50% or 80% formamide. Probes were used in hybridisation reactions on the day of

their synthesis, since high specific activity ^{32}P-labelled probes rapidly break down due to radiochemical damage (Zinn *et al.* 1983).

Samples, standards and controls were placed in microcentrifuge tubes and the mass of RNA was adjusted to 20µg with yeast transfer RNA (tRNA). 2.5 volumes of ethanol and 0.1 volume of 3M sodium acetate (pH5.5) were added and the RNA was allowed to precipitate at -20C for at least 1h. All assays included one or two background control tubes, which contained only the tRNA. Samples were centrifuged and the pellets washed with 70% ethanol and dried under vacuum.

The samples were then dissolved in 20µl of the probe solution by gentle vortexing and heating to 65C for 10min. The same volume of probe solution was also added to a tube containing no RNA, to act as an input control. The samples were then brought to the bottom of the tubes by brief centrifugation and covered with two drops of mineral oil (Sigma). RNA was denatured by heating the tubes to 90C for 5min before being transferred to a water bath at the appropriate hybridization temperature. A range of different hybridisation temperatures were tested; 75C, 80C and 85C for hybridisation in the absence of formamide; 60C, 65C and 70C for hybridisation in 50% formamide; 45C, 50C and 55C for hybridisation in 80% formamide, based on hybridisation temperatures used by other workers (Leung *et al.* 1987; Lynn *et al.* 1983; Melton *et al.* 1984). Hybridization was allowed to take place for at least 12h.

After hybridization, RNases A and T_1, in 300µl of an ice-cold solution containing 10mM **Tris** (pH7.5), 300mM NaCl and 5mM EDTA were added to all tubes except the input controls. Many published protocols have used concentrations of 40µg/ml RNase A and 2µg/ml RNase T_1 (Melton *et al.* 1984; Zinn *et al.* 1983). Therefore, the following concentrations of RNase were tested for their effect on the efficiency of the assay:

	RNase A (µg/ml)	RNase T_1 (µg/ml)
5x	200	10
2x	80	4
1x	40	2
0.5x	20	1
0.2x	8	0.4

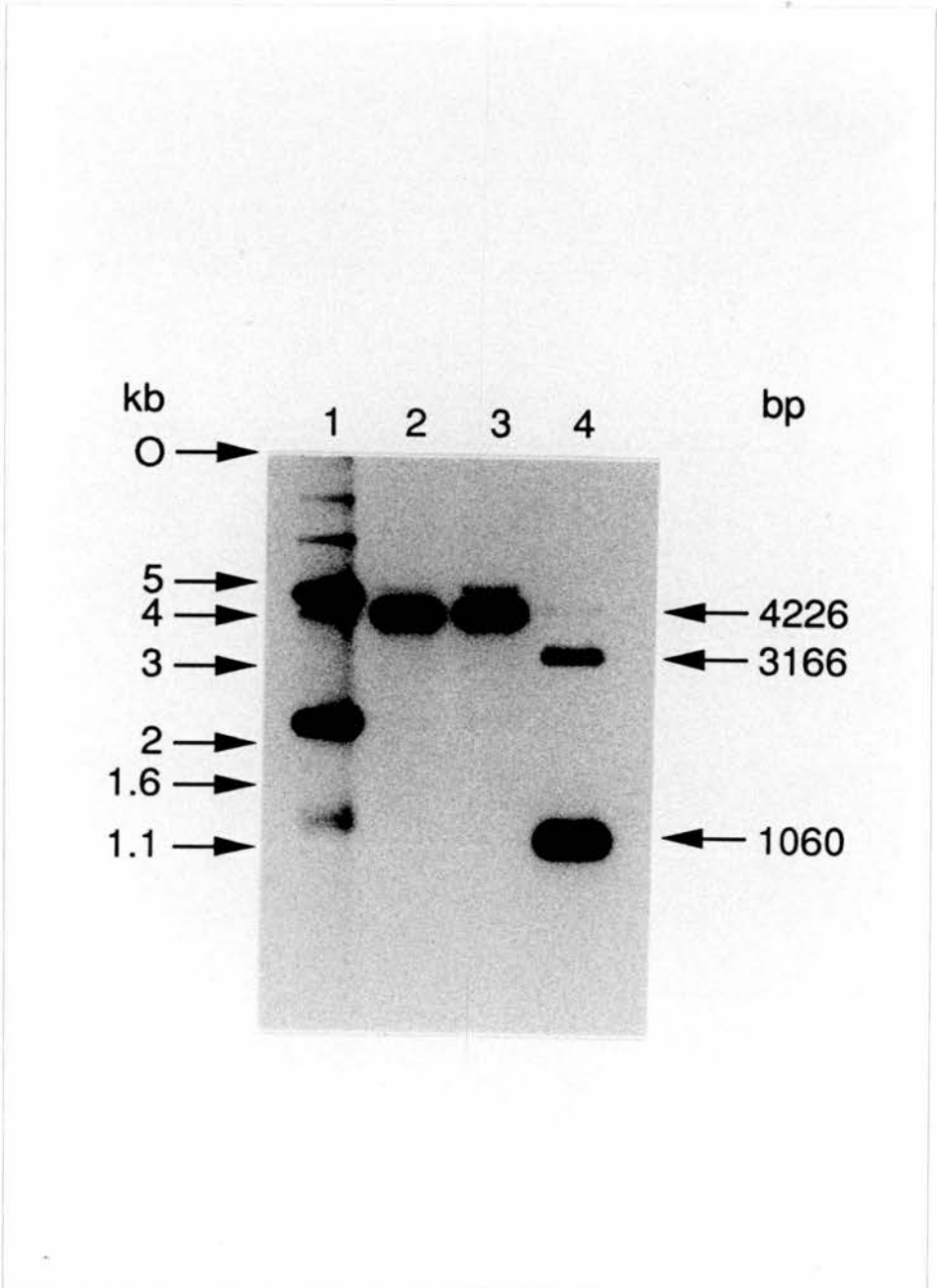
Tubes were incubated at 32C for 1h. In order to destroy the RNase, 100µg of proteinase K and 10µl 20% SDS were added to all tubes except input controls, and tubes were incubated for a further 15min at 37C. The input controls were adjusted to 320µl, all samples were extracted once with phenol:chloroform (1:1), and 300µl of the aqueous phases transferred to fresh tubes. Samples were precipitated at -20C for at least 1h with 30µl 3M sodium acetate (pH5.5) and 750µl cold ethanol, in the presence of 20µg carrier yeast transfer RNA. RNA was pelleted and redissolved in 20µl sterile water. To visualise protected probe, samples were analysed by polyacrylamide gel electrophoresis and autoradiography as described above. Only one tenth of the input control samples were loaded on gels. To measure the amount of probe protected by increasing amounts of synthetic sense RNA, duplicate 5µl aliquots of each sample were spotted onto DE81 filters, washed and subjected to liquid scintillation counting as described in chapter 2, section 2.6.4.

3 Results

3.1 Subcloning

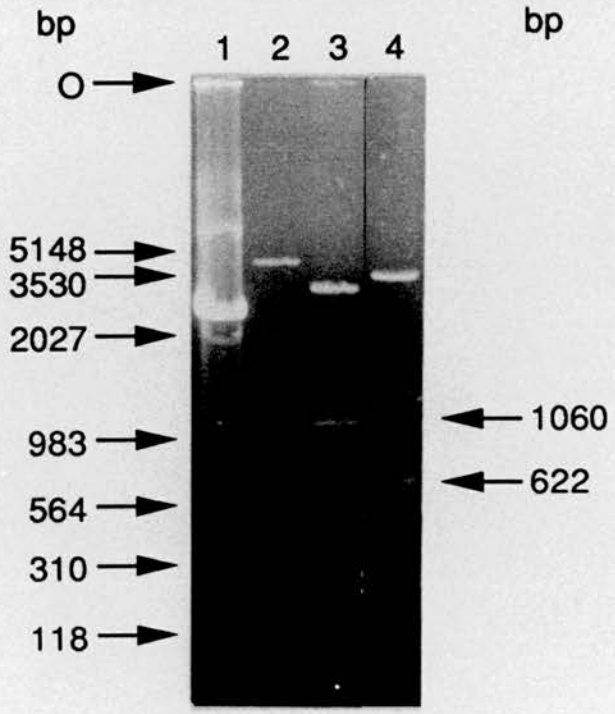
Fig.5.5 shows a Southern blot of plasmid prepared from a clone of *E. coli* transfected with a product of the ligation described above. Digestion with either *Eco*RI or *Sal*I yielded a single band, as expected, and digestion with both enzymes liberated an insert of approximately 1kb from a plasmid migrating at the expected molecular weight of ~3.2kb. The insert hybridised strongly to the inhibin-α cDNA probe, and it was concluded that this insert consisted of the 1027bp inhibin α-subunit cDNA fragment, plus 33bp of the multiple cloning site of the plasmid. Although some hybridisation of the probe to the ~3.2kb band was observed, it was of much lower intensity than that seen to the insert, and was therefore considered to be non-specific. Further analysis of this clone confirmed that digestion with *Eco*RI and *Sal*I liberated the expected

Fig.5.5. Southern analysis of a positive clone containing the 1027bp fragment of the inhibin-α cDNA. Plasmid and restriction digests were separated by electrophoresis in a 0.8% agarose gel, and DNA was transferred to a nylon membrane. The blot was probed with a ^{32}P-labelled rat inhibin α-subunit cDNA probe, washed at 65C, and exposed to x-ray film for 30min. Lane 1: undigested plasmid. Lane 2: plasmid digested with *Eco*RI. Lane 3: plasmid digested with *Sal*I. Lane 4: plasmid digested with both *Eco*RI and *Sal*I. Positions of molecular weight standards are marked on the left.


kb
1
2
3
4
bp
O
5
4
3
2
1.6
1.1
4226
3166
1060

1060bp insert, and also revealed that digestion with *Eco*RI and *Bgl*II excised a 622bp fragment of the insert (Fig.5.6), indicating that the insert was cloned into the multiple cloning site of the plasmid in the orientation shown in Fig.5.7. Preparation of unlabelled RNA from plasmid linearised with *Bgl*II generated a 630 base transcript using T7 RNA polymerase, and a 466 base transcript using SP6 RNA polymerase (Fig.5.8). The sizes of these transcripts are consistent with the orientation of the insert as shown in Fig.5.7. Transcription of RNA from plasmid linearised with *Eco*RI using SP6 RNA polymerase yielded a transcript 1091 nucleotides in length, as expected. These results showed that the subcloning of the 1027bp fragment of the rat inhibin α-subunit cDNA was successful, and that the orientation of the insert in this construct was such that antisense RNA probes could be generated using T7 RNA polymerase, and sense (standard) RNA could be prepared using SP6 RNA polymerase. Hybridisation of presumptive antisense RNA prepared using T7 polymerase to granulosa cell RNA is shown in Fig.5.9A. The probe detected a major mRNA species of ~1.7kb, the levels of which were highest in granulosa cells from animals which had received FSH *in vivo*, and reduced in the granulosa cells of those which had received hCG following FSH treatment. The molecular weight and pattern of expression of this species are entirely consistent with those of inhibin-α mRNA as detected using cDNA probes (see previous chapter, Fig.4.2). A considerable amount of non-specific binding of this probe to the 28s ribosomal RNA band was observed in this case, although the intensity of hybridisation was far less great than to the inhibin α-subunit mRNA. As expected, a probe synthesised from the opposite strand did not hybridise to inhibin-α mRNA under the same conditions (Fig.5.9B). However, and most unexpectedly, this probe did hybridise to a low molecular weight species (~600 bases), the abundance of which was markedly stimulated by treatment of animals with hCG *in vivo*, and unaffected by treatment with FSH.

Fig.5.6. Restriction analysis of the positive clone shown in Fig.5.5. Plasmid was digested with *Eco*RI (lane 2), *Eco*RI and *Sal*I (lane3) and with *Eco*RI and *Bgl*II (lane 4). Lane 1 contains undigested plasmid. DNA was resolved by electrophoresis in a 0.8% agarose gel containing ethidium bromide. Positions of molecular weight standards are marked on the left.


bp
1 2 3 4
bp
O
5148
3530
2027
983
564
310
118
1060
622

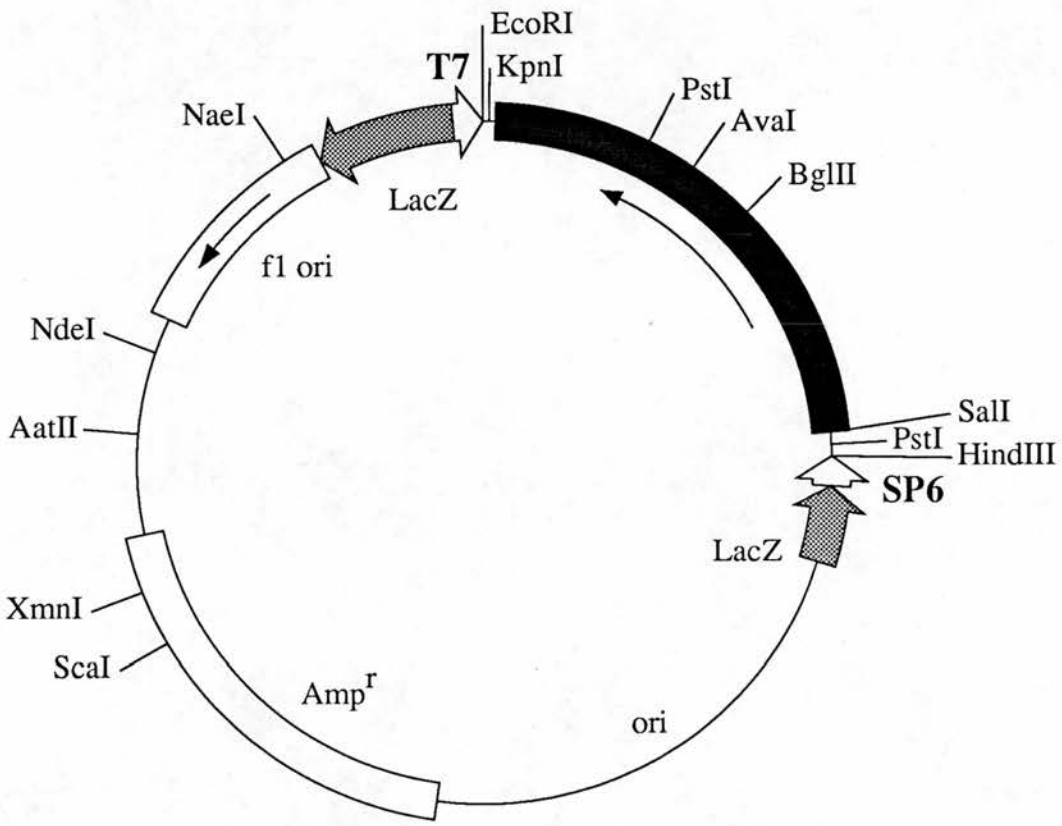


Fig.5.7. Map of the positive clone shown in Figs.5.5 and 5.6. The 1027bp rat inhibin α-subunit insert is marked in black, and its orientation with respect to the sense strand (5'-3') is marked with an arrow below. Plasmid features are marked as in Fig.5.2, except the SP6 and T7 promoters, which are marked as unfilled arrows, and only selected restriction sites in the multiple cloning site are shown.

Fig.5.8. Transcription *in vitro* using subcloned inhibin-α cDNA as a template. Lane 1: RNA molecular weight markers, the sizes of which (in kb) are marked on the left. Lane 2: RNA transcribed from plasmid digested with *Bgl*II, using T7 RNA polymerase. Lane 3: RNA transcribed from plasmid digested with *Bgl*II, using SP6 RNA polymerase. Lane 4: RNA transcribed from plasmid digested with *Eco*RI, using SP6 RNA polymerase. RNA was resolved by electrophoresis in a 4% denaturing polyacrylamide gel, and stained with ethidium bromide.

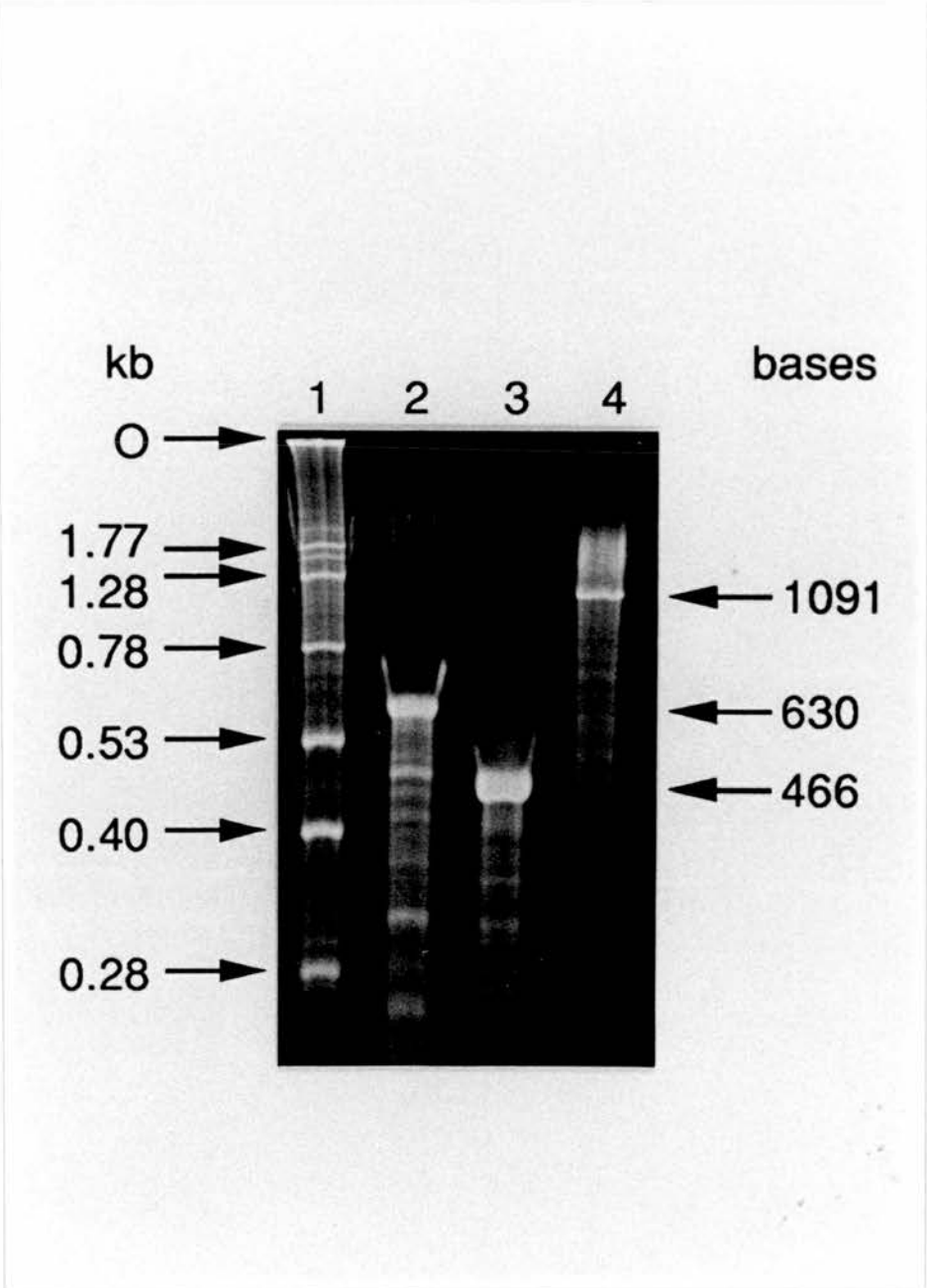
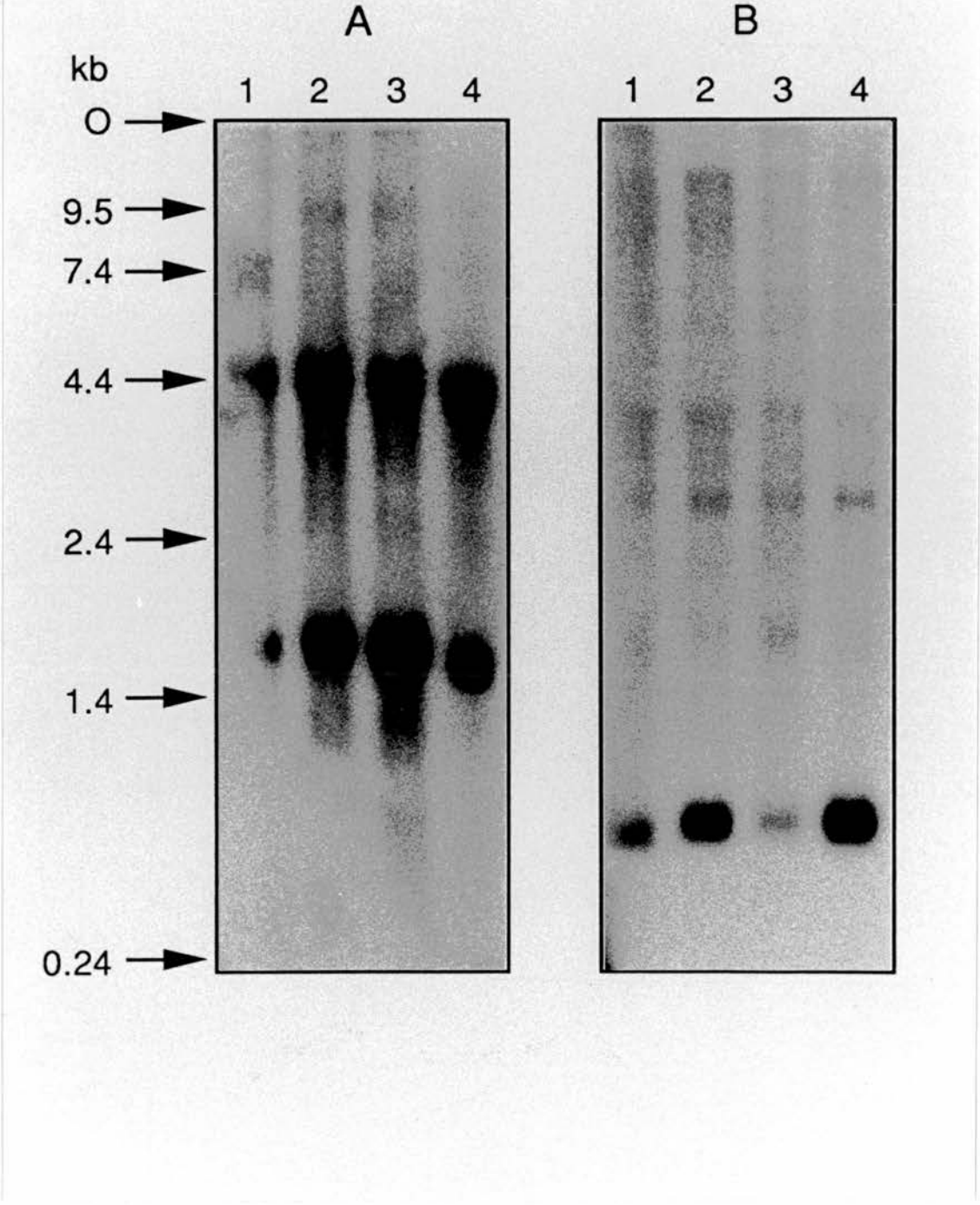

kb
bases
1 2 3 4
O
1.77
1.28
0.78
0.53
0.40
0.28
1091
630
466

Fig.5.9. Northern hybridization of ^{32}P-labelled RNA probes to total RNA from rat granulosa cells. Probes were transcribed from plasmid containing the 1027bp fragment of the inhibin-α cDNA, linearized with *Bgl*II, using T7 (panel A) or SP6 (panel B) RNA polymerases. Probes were hybridized to identical Northern blots bearing 20μg of total RNA per lane from granulosa cells of untreated rats (lane 1), or rats treated *in vivo* with 20IU hCG (lane 2), 4 injections of 40μg oFSH (lane 3), or both FSH and hCG (lane 4). Blots were washed twice for 30min at 65C, and exposed to x-ray film for 10h at -70C.


A
B
kb
1
2
3
4
1
2
3
4
O
9.5
7.4
4.4
2.4
1.4
0.24

3.2 Factors Affecting the Efficiency of RNase Protection

The results of an experiment to determine the effect of different concentrations of formamide and different hybridisation temperatures on the efficiency of hybridisation in this assay are shown in Fig.5.10. In all three hybridisation buffers used (0%, 50% and 80% formamide) hybridisation temperature did not appear to affect greatly the efficiency of hybridisation across the narrow range tested. However, the concentration of formamide in the buffer used did have a marked effect. In a buffer containing no formamide, probe protected by hybridisation with sense RNA was readily detected by overnight exposure of gels. Some low molecular weight smearing was present, although this was also present in the probe preparation, and was probably due to radiochemical breakdown products of the probe (Zinn *et al.* 1983). No RNase-resistant probe was present in the absence of sense RNA. However, where the hybridisation buffer contained 50% formamide, protected probe was only adequately detected after exposure of gels for 8 days. The degree of low molecular weight smearing was greater than that present in the probe preparation, indicating incomplete protection, and some probe was detected in the background controls after this exposure time. In a buffer containing 80% formamide, the amount of probe protected was barely above background. The effect of different concentrations of RNase in the digestion mixture on yield of protected probe was also investigated (Fig.5.11). It was found that the yield of protected RNA was greatest at the lowest concentration of RNase used (8µg/ml RNase A, 0.4µg/ml RNase T_1), which is much lower than that used in most published protocols (Leung *et al.* 1987; Melton *et al.* 1984; Sambrook *et al.* 1989). At this concentration of RNase, no probe was detected in background controls after overnight exposure of gels.

The method adopted for subsequent RNase protection assays was therefore to hybridise in a buffer containing no formamide at a temperature of 75C, and to digest unhybridised probe in a buffer containing 8µg/ml RNase A and 0.4µg/ml RNase T_1.

Known amounts (25-100pg) of synthetic sense RNA were hybridised to a ^{32}P-labelled probe using the method described above. After digestion

Fig.5.10. Influence of hybridization temperature and formamide concentration on RNase protection of ^{32}P-labelled inhibin-α cRNA probe by synthetic sense RNA. Formamide concentrations in the hybridization buffers were as follows. Panel A: no formamide; panel B: 50% formamide; panel C: 80% formamide. Hybridization temperatures were as follows. Panel A, lanes 3, 4 and 5: 75, 80 and 85C, respectively; panel B, lanes 3, 4 and 5: 60, 65 and 70C, respectively; panel C, lanes 3, 4 and 5: 45, 50 and 55C, respectively. In each panel, an input control (probe only, no RNase treatment; lane 1) and a background control (probe only, digested with RNase; lane 2). Probe ($2x10^5$cpm; ~1ng) was allowed to hybridise to 100pg synthetic sense RNA for 16h, and unhybridised probe was digested with 40μg/ml RNase A and 2μg/ml RNase T_1. Protected fragments were analyzed by electrophoresis in 4% (panels A and B) or 6% (panel C) polyacrylamide gels containing 7M urea, followed by autoradiography. Exposure times were: panel A, 24h; panel B, 8d; panel C, 17d.

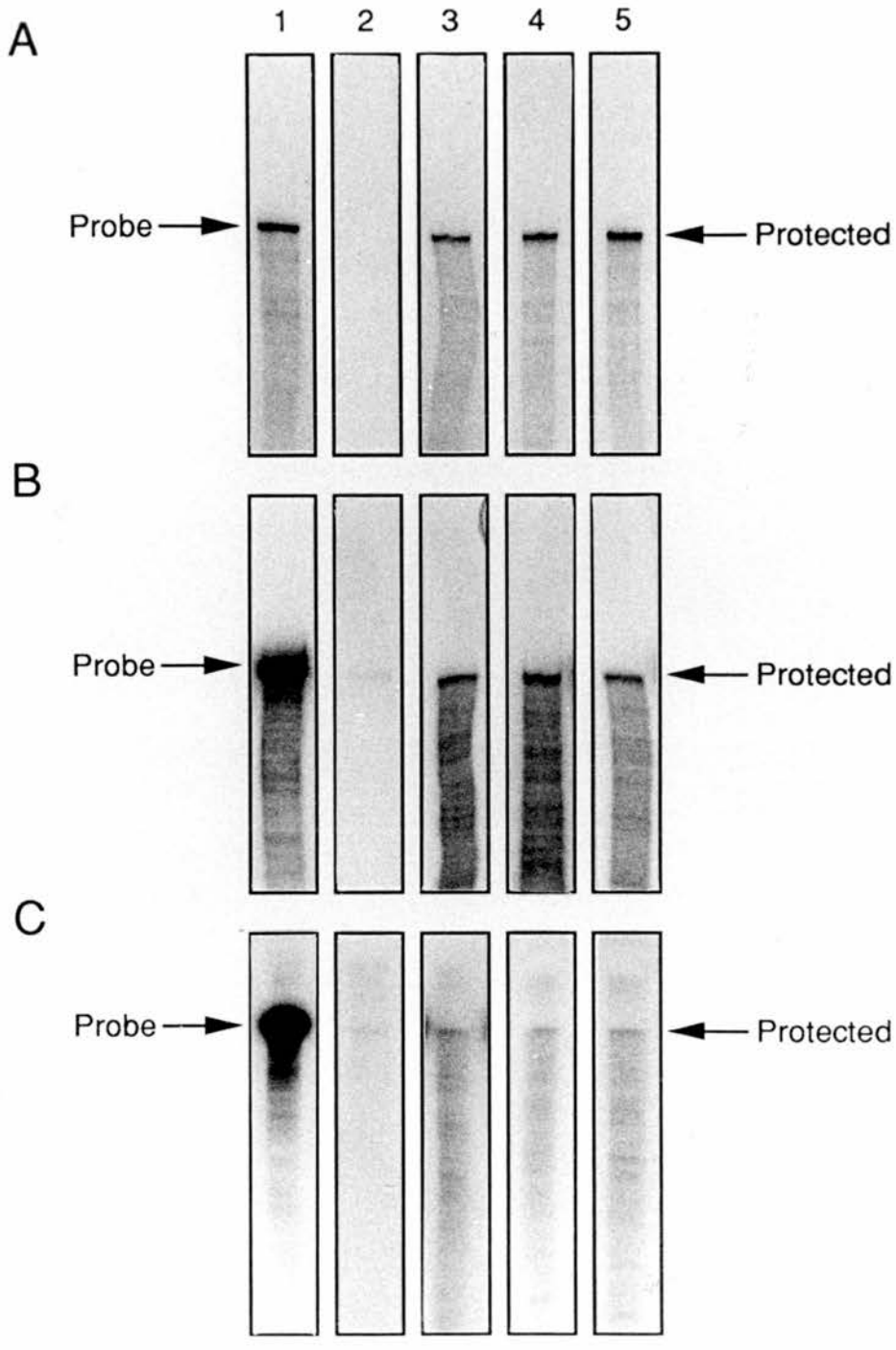
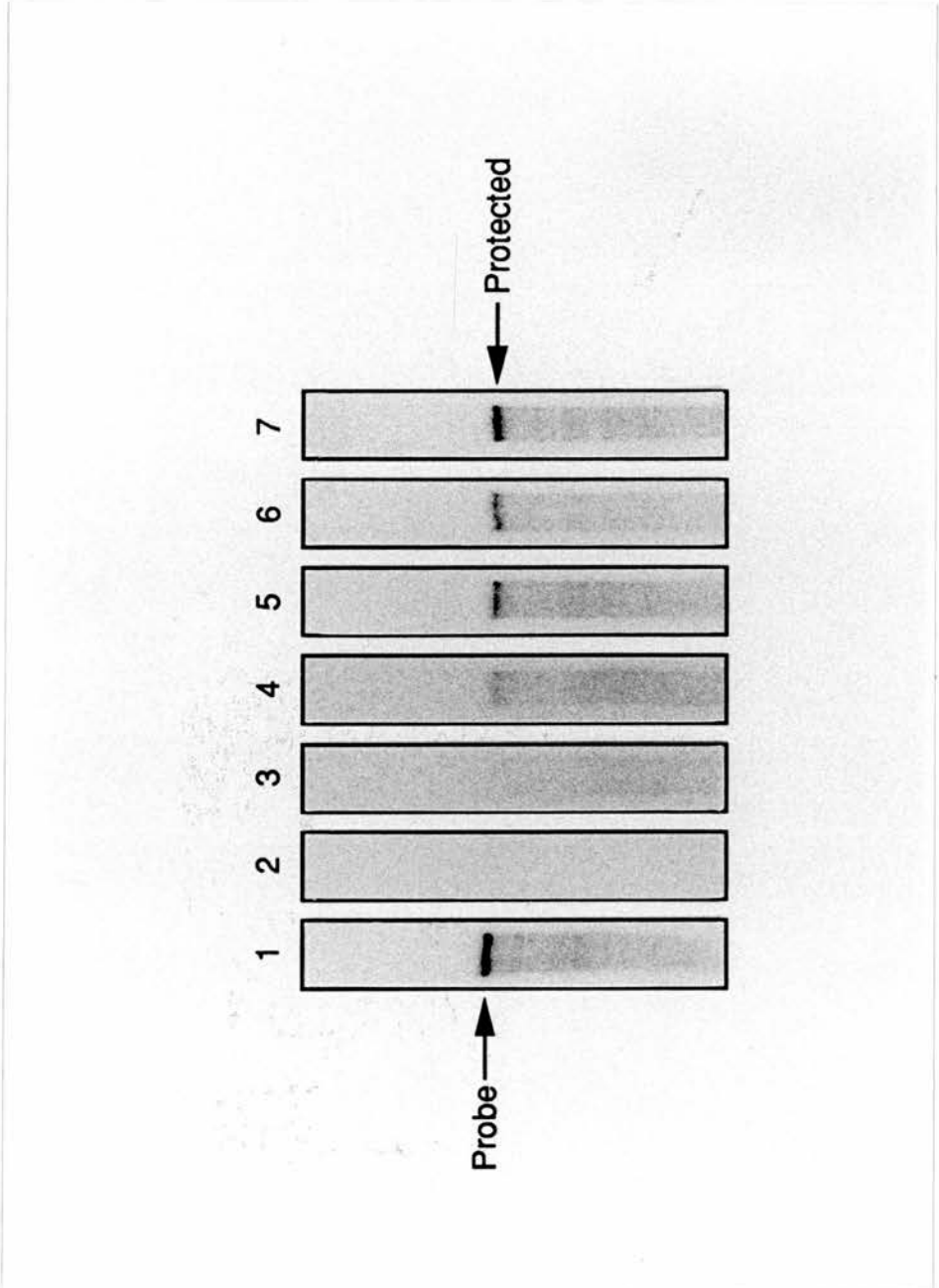

A
1
2
3
4
5
Probe
Protected
B
Probe
Protected
C
Probe
Protected

Fig.5.11. Resistance of RNA hybrids to different concentrations of RNase. $2x10^5$ cpm of ^{32}P-labelled cRNA probe was hybridized to 100pg of synthetic sense RNA for 16h at 75C in a formamide-free buffer. Samples were treated with the following concentrations of RNases: lane 3, 200µg/ml RNase A, 10µg/ml RNase T_1; lane 4, 80µg/ml RNase A, 4µg/ml RNase T_1; lane 5, 40µg/ml RNase A, 2µg/ml RNase T_1; lane 6, 20µg/ml RNase A, 1µg/ml RNase T_1; lane 7, 8µg/ml RNase A, 0.4µg/ml RNase T_1. An input control (probe only, no RNase treatment; lane 1) and a background control (probe only, digested with 8µg/ml RNase A and 0.4µg/ml RNase T_1; lane 2) were also included. Resistant fragments were visualized by electrophoresis in 4% polyacrylamide gels containing 7M urea, followed by autoradiography. Exposure was for 24h.


Protected
7
6
5
4
3
2
1
Probe

with RNases, protected probe in duplicate aliquots of each sample was adsorbed to DE81 filters and measured by liquid scintillation counting. The resulting standard curve is shown in Fig.5.12. It was found that detectable protection was achieved by the lowest amount (25pg) of standard RNA used in this assay. Therefore, another standard curve was constructed, using a wider range of standards (4-125pg). In the same assay, probe was also hybridised to 10μg of total ovarian RNA from control animals and from animals treated with FSH *in vivo*. Protection of probe was assessed by electrophoresis and autoradiography (Fig.5.13). It was found that 8pg of synthetic sense RNA was detected after overnight exposure of the gels (Fig.5.13A), and that, by comparison with standards, ovaries from control animals contained inhibin-α mRNA levels equivalent to approximately 60pg standard. Ovarian RNA from FSH-treated animals contained approximately twice that amount. Unfortunately, in that experiment, the background of radioactivity, visible on the gels as a dark smear, was very high, so that scintillation counting of samples did not reveal any differences between the samples. This background did not indicate incomplete protection of probe, since it was of both higher and lower molecular weight than the probe, and was present in equal amounts in samples and controls.

4 Discussion

The aim of the part of the project described in this chapter was to develop and validate a sensitive, quantitative and reproducible RNase protection assay for the measurement of inhibin α-subunit mRNA, and to use this assay to study in detail the control of expression of this mRNA. To this end a portion of the rat inhibin α-subunit cDNA was successfully subcloned into a suitable vector, and synthetic RNA probes and sense standards transcribed from this construct were used to perform the preliminary stages of the development of an RNase protection assay. It was confirmed that specific RNA levels can be quantified by scintillation counting of protected probe bound to charged paper filters, and a linear relationship appeared to exist between the counts of probe detected by this method and the amount of sense RNA to which the

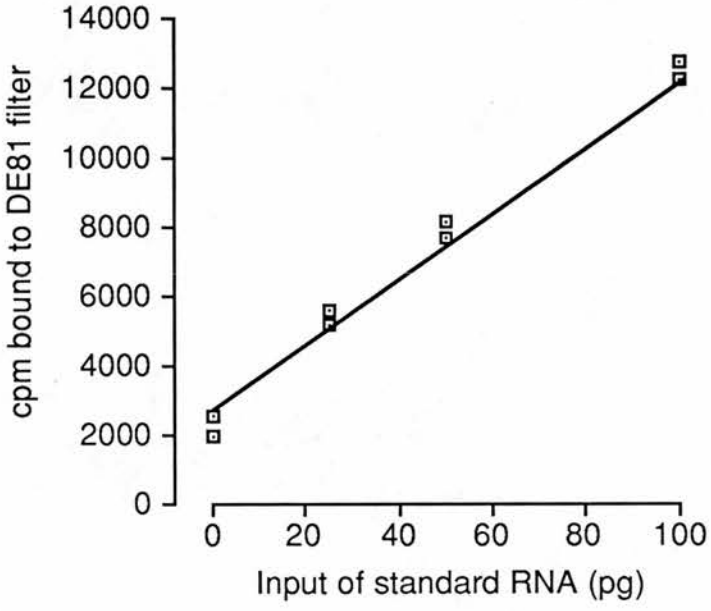
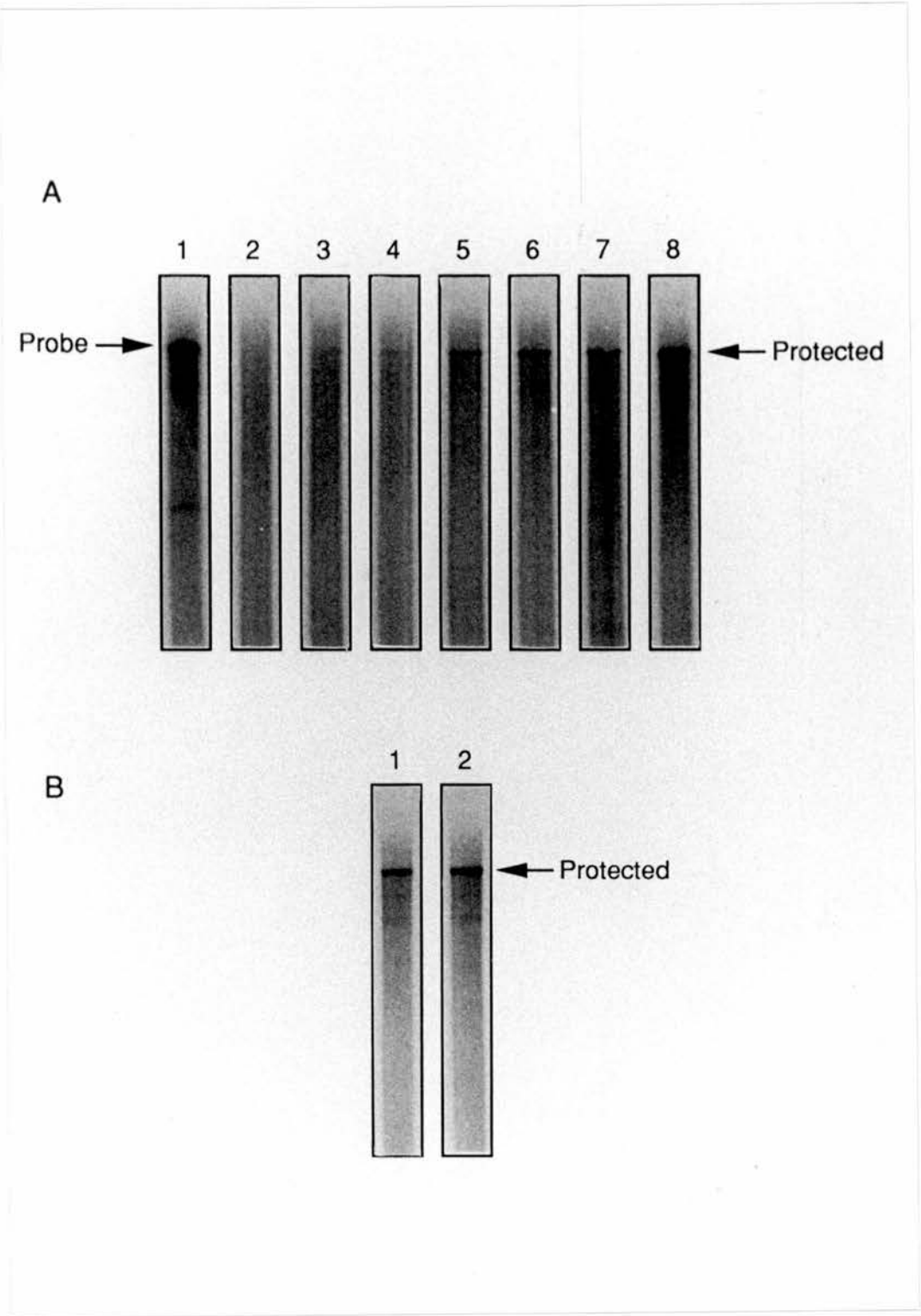


Fig.5.12. Quantitation by liquid scintillation counting of inhibin-α cRNA probe protected by increasing amounts of synthetic sense RNA. $2x10^5$cpm ^{32}P-labelled probe was hybridized with 25, 50 or 100pg of sense RNA for 16h at 75C in formamide-free hybridization buffer, before digestion with 8μg/ml RNase A and 0.4μg/ml RNase T_1. Duplicate 5μl aliquots of each samples were applied to DE81 filters, washed four times with 0.5M Na_2HPO_4 (pH7), rinsed in 70% ethanol and dried. Filters were placed in scintillation vials with 4ml scintillation fluid, and emissions counted on the ^{32}P channel of a scintillation counter.

Fig.5.13. Visualisation of inhibin-α cRNA probe protected by increasing amounts of synthetic sense RNA, and by inhibin-α mRNA in rat ovarian RNA samples. Panel A: lane 1, input control; lane 2, background control; lanes 3-8, $2x10^5$cpm of ^{32}P-labelled antisense inhibin-α probe was hybridized to 4 (lane 3), 8 (lane 4), 16 (lane 5), 31 (lane 6), 62 (lane 7) or 125pg (lane 8) sense RNA. Panel B: $2x10^5$cpm of inhibin-α cRNA probe was hybridized to its target mRNA in 10μg of total RNA extracted from ovaries of immature untreated rats (lane 1), or rats treated *in vivo* with 4 injections of 20μg oFSH (lane 2). Samples were digested with 8μg/ml RNase A and 0.4μg/ml RNase T_1. RNase-resistant hybrids were detected by electrophoresis and autoradiography.


A
1
2
3
4
5
6
7
8
Probe
Protected
B
1
2
Protected

probe had been hybridised. The sensitivity of the assay as determined by autoradiography of protected probe appeared to be less than 8pg, although the background level of non-specific radioactivity in the assay from which that estimate was obtained was very high, so that differences in probe protection between samples were difficult to detect. This is much below the level of sensitivity claimed for this type of assay by other workers (Hames & Higgins, 1987; Melton *et al.* 1984), and it is not clear whether the assay described in the foregoing section is any more sensitive than Northern analysis, since no direct comparison was made. However, apparent levels of mRNA encoding inhibin α-subunit detected using this method suggest that the assay would be capable of detecting this message in less than 1μg of total ovarian RNA.

Clearly, for the successful application of this assay, more rigorous studies of the effects of hybridisation temperature, buffer, RNase concentration and length and specific activity of probes on the sensitivity of the assay would be required, as well as a direct comparison between RNase protection and Northern analysis. However, due to constraints of time, this work was beyond the scope of the present thesis.

An interesting finding to emerge from the work described in this chapter was the detection of an RNA species abundantly expressed in granulosa cells which hybridised strongly to the sense RNA probe. Because the blot was washed at a high stringency, this RNA appeared to share considerable homology with the non-coding strand of the inhibin-α cDNA. However, this molecule was not detected using double-stranded cDNA probes (see previous chapter), suggesting that this sequence homology is not complete.

The function of such a molecule is unknown, although functions for antisense RNA have been proposed in other systems. Antisense RNA has been most closely studied in prokaryotic systems, including plasmids, bacteriophages, transposons and bacteria, where they inhibit translation, lead to termination of transcription, or affect the stability of specific mRNAs (for reviews, see Eguchi, *et al.*, 1991, and Simons & Kleckner, 1988). A few cases of naturally occuring antisense RNA expression in eukaryotes have recently been reported (Simons, 1988; Mol *et al.* 1990). Most strikingly, a gene encoding a novel member of the nuclear

hormone receptor superfamily, similar to the receptors for retinoic acid and thyroid hormones, was recently cloned. Although the protein encoded by this gene exhibits some binding affinity for triiodothyronine (Miyajima *et al.* 1989), its true ligand has not yet been identified. Remarkably, this gene was found to overlap the c-*erb*A gene, which encodes the thyroid hormone receptor, but its mRNA is transcribed from the opposite strand (Lazar *et al.* 1989; Miyajima *et al.* 1989). Similarly, both strands of parts of the N-*myc* (Krystal *et al.* 1990) and c-*myc* (Piechaczyk *et al.* 1988; Nepveu *et al.* 1987) genes are transcribed. In the case of N-*myc*, at least, the antisense RNA hybridises to the mRNA *in vivo*, preventing splicing of the first intron (Krystal *et al.* 1990). Antisense transcription from the IGF-II gene has also been demonstrated in chicks (Taylor *et al.* 1991). In all these cases, levels of antisense RNA were of the same order as levels of the corresponding mRNA. Strong evidence of regulation of mRNA processing by antisense RNA has also been found in the case of bFGF expression in *Xenopus* oocytes. An antisense transcript of this gene has been cloned, which encodes an unknown protein, and is also complementary to the coding region of the bFGF mRNA (Volk *et al.* 1989). Furthermore, during maturation of the oocyte, this antisense RNA binds to the bFGF mRNA, leading to conversion of adenine residues to inosine in the region of hybridisation (Kimelman & Kirschner, 1989). The authors postulate a role for this mechanism in the regulation of bFGF mRNA stability. Also, genetic myelin deficiency in mice has been attributed to inhibition of myelin basic protein mRNA translation by antisense RNA transcribed at a high rate from an inverted tandem duplication of part of this gene (Tosic *et al.* 1990).

An inhibitory role for naturally occuring antisense RNA is supported by the applicability of transfected or microinjected antisense nucleic acids in the inhibition of translation of specific mRNA (Green *et al.* 1986; van der Krol *et al.* 1988). However, another intriguing possibility is that antisense RNA may encode proteins with properties related to those of the corresponding product of the sense mRNA. It has been shown that, in some cases, peptides translated from complementary RNA sequences exhibit high affinity binding for one another (Blalock, 1990), suggesting profound implications for the molecular basis of ligand-

receptor interactions (Blalock & Bost, 1988). The observation that RNA complementary to angiotensin II mRNA encodes a specific angiotensin II receptor antagonist (Moore *et al.* 1989) is consistent with this hypothesis. Therefore, the interesting possibility arises that the species detected with the inhibin-α sense probe could encode an inhibin binding protein, antagonist, or even receptor. However, this seems unlikely, since the sense probe used here was generated from a part of the cDNA encoding the pro- and αN regions of the inhibin α-subunit precursor protein (see Fig.5.3), which are not present in the mature subunit. Since the region of homology between the probe and this species has not been determined, this does not mean that the "antisense" RNA is not complementary to other portions of the inhibin-α mRNA.

Based on the sequence similarity between the coding strands of a number of receptor genes and the antisense strands of other genes, it has been proposed that one mechanism whereby new proteins evolve is by utilisation of the noncoding strands of preexisting genes (Kunisawa & Otsuka, 1987). This hypothesis implies that apparent antisense transcription from a particular gene may not have any relevance to the control of expression of that gene, but may simply indicate expression of a distinct gene which arose from the use of sequences already existent in the noncoding strand of that gene. The protein product of a gene arising in this way need not be related in any other way to the product of the parent gene.

Based purely on the weight of literature suggesting that antisense RNA functions directly to interfere with the processing of the corresponding mRNA, rather than the two latter explanations discussed above, the most likely interpretation of the detection of "antisense" inhibin-α RNA is that it acts to suppress inhibin-α production at some level of RNA processing. If this is the case, it adds another level of complexity to the already confusing subject of the control of inhibin synthesis. The hormonal regulation of the expression of this species was also interesting. FSH treatment *in vivo* had no effect on levels of this transcript in granulosa cells, but hCG, with or without prior FSH treatment, caused a substantial increase in its abundance. Since hCG causes a drop in levels of inhibin-α mRNA after prior FSH treatment (see

chapter 4, Figs. 4.2 and 4.6), and an increase in levels of "antisense" inhibin-α RNA, this may constitute a dual mechanism to explain the fall in inhibin production by large follicles which occurs at the LH surge (Yohkaichiya *et al.* 1991; Rivier *et al.* 1989; Watanabe *et al.* 1990). This absence of effect of FSH, either on the levels of this transcript or on the effect of hCG thereon, strongly suggests that the expression of this molecule is under the control of a thecal cell product, whose production is stimulated by hCG. The most obvious, although by no means only, candidate for such a role is androgen. It would be very interesting to study the effects of gonadotrophins and theca-derived steroids and growth factors on the levels of this "antisense" RNA under the defined conditions of the *in vitro* system used in this thesis.

Further characterisation of this "antisense" RNA species will be required before its significance is revealed. The region of homology shared by this transcript and the inhibin α-subunit mRNA could easily be determined by the use of probes transcribed from different regions of the inhibin-α cDNA, and the degree of this homology could be determined by measuring the temperature at which cross-hybridising inhibin-α sense probes are dissociated from the "antisense" RNA. However, in order to ascertain the nature of this molecule, it would be necessary to isolate its cDNA. It would also be necessary to test the hypothesis that it is capable of binding to inhibin-α mRNA *in vivo*. Of course, it would also be interesting to investigate whether equivalent antisense RNAs exist for the inhibin β-subunit messages. Such aims are, unfortunately, beyond the scope of the current project.

Chapter 6 General Discussion

1 Value of the DES-Primed Rat Granulosa Cell Model

In order to study the control of a biological system at any level, a simplified model must be employed. Such a model must be simplified to the extent that any observations can be unequivocally ascribed to the influence of the test treatment, and so in determining the role of one factor, many other factors inevitably have to be ignored. No *in vitro* model can mimic perfectly the conditions present *in vivo*, and it is useful only if it retains some similarity to those conditions. The rat granulosa cell culture model used throughout the experiments described in this thesis was designed to provide a convenient means of studying the role of sex steroids in the paracrine control of protein synthesis and gene expression which accompany granulosa cell differentiation. Because the cells were isolated from their normal environment, some hormonal and physical factors, which are certain to affect the function of granulosa cells *in vivo*, could not be evaluated. However, since similar model systems have been widely used in the past in the study of the local control of follicular development and function (Dorrington *et al.* 1975; Sanders & Midgley, 1982; Hsueh *et al.* 1984), it was considered to be suitable for studies of the intraovarian role of sex steroids. It has long been known that administration of oestrogen to rats *in vivo* causes increased ovarian weight as a result of the development of large numbers of large preantral and small antral follicles (Simpson *et al.* 1941) due to proliferation of the granulosa cells (Goldenberg *et al.* 1972). It is this property which has been exploited in this culture model to provide a convenient means of isolating large numbers of relatively undifferentiated granulosa cells which can be induced to differentiate in culture in response to FSH. It is still not known how oestrogen exerts this effect, and it is interesting that oestrogen treatment *in vivo* stimulates granulosa cell IGF-I, IGF-I

receptor and TGF-β mRNA expression (Hernandez *et al.* 1990b; Hernandez *et al.* 1991; Mulheron & Schomberg, 1992), and the production of a thecal cell factor which is mitogenic for granulosa cells (Bley *et al.* 1991).

Since the aim of the studies described in this thesis was to study the roles of steroids in granulosa cell differentiation, it was important that pretreatment of cells with oestrogen did not compromise their subsequent responsiveness to steroids. Several studies have shown that pretreatment of animals with oestrogen increases the subsequent effects of FSH *in vitro* on cAMP formation (Jonassen *et al.* 1982), RIIβ expression (Hedin *et al.* 1987a), aromatase activity and gene expression (Bogovich & Richards, 1984; Fitzpatrick & Richards, 1991), side-chain cleavage expression (Goldring *et al.* 1987) and LH receptor formation (Richards *et al.* 1976; Segaloff *et al.* 1990). It has also been suggested that prolonged exposure to high levels of oestrogen *in vivo* can reduce the capacity of granulosa cells to proliferate *in vitro* (Chakravorty *et al.* 1991). However, data presented in Figs.2.24, 3.6 and 4.12 showed no marked effect of DES pretreatment on the semi-quantitative end-points of patterns of protein synthesis and gene expression. Some enhancement by DES of the effect of FSH and testosterone on progesterone production was observed (Fig.2.23), but the overall pattern of hormonal responsiveness was unaffected.

The major potential artefact of using this culture model identified in the results presented here was the apparent morphological differentiation of granulosa cells cultured on plastic dishes. The most striking differences observed between cultured and freshly isolated granulosa cells were in their patterns of protein synthesis, which appeared to change with time and plating density in culture (Chapter 2). One of the proteins most markedly dependent upon time and cell density in culture was fibronectin (see Chapter 3), which is involved in cell spreading and attachment. Since this protein was not synthesised by freshly isolated cells, it is likely that at least some of the changes in protein synthesis observed during culture reflected structural adaptations of the cells to culture. Therefore, some of the observed effects of FSH on protein synthesis by cultured granulosa cells probably reflected a reversal of these adaptations necessary for the expression of the functional effects of FSH.

The relatively slower time-course of stimulation by FSH of functional markers of granulosa cell differentiation (see Figs.2.12, 4.8 and 4.9), when compared to that of the suppression of actin mRNA levels by FSH (see Fig.3.7), also supports this interpretation. A similar inverse relationship between synthesis of cytoskeletal and extracellular matrix proteins and markers of differentiated function has been shown before (Skinner *et al.* 1985; Ben Ze'ev & Amsterdam, 1986). Similarly, the absence of any effect of gonadotrophins on actin expression *in vivo* argues strongly against a physiological role for these morphological changes *in vivo*. The very low basal level of expression of inhibin-α mRNA in culture (see Fig.4.6), when compared to freshly isolated cells (see Fig.4.2), may also be due to constraints imposed by structural adaptation of these cells to culture. These observations suggest that this model is not suitable for the study of structural aspects of granulosa cell differentiation.

In order to determine the extent to which granulosa cells in culture behave functionally as they do *in vivo*, the responses of granulosa cells to gonadotrophins were evaluated both *in vitro* and *in vivo*. The effects of gonadotrophins on the expression of mRNA encoding the functional markers of differentiation studied (i.e. inhibin subunits, the LH receptor, P450arom and P450scc, see Chapter 4) were qualitatively the same *in vivo* and *in vitro*. The exception to this was the effect of hCG alone. In cultured cells, hCG was never observed to have any effect on expression of any of the genes studied, and did not affect progesterone production *in vitro*. However, when administered *in vivo*, hCG had a slight stimulatory effect on expression of P450arom, P450scc and LH receptor, and a strong stimulatory effect on the expression of the inhibin subunits. It could be argued that this effect of hCG was mediated via the theca, which would be expected to have LH receptors in all follicles. The absence of any effect of hCG alone on cultured cells would seem to support this interpretation. However, this effect was variable between experiments. This implies that the granulosa cells of the animals were not as immature as one might expect, having some degree of LH responsiveness, and that the state of maturity of cells obtained using this model is not always the same. This may be due to variable levels of pituitary function between individuals, or variable efficacy of DES

capsules, allowing some gonadotrophin stimulation to reach the ovary. The obvious solution to this problem would be to use hypophysectomised animals, but the cost of routinely using hypophysectomised animals is unjustifiable.

Although cultured granulosa cells are morphologically different from those in a follicle, and respond relatively slowly to hormonal stimuli, these cells can be induced to differentiate functionally, as indicated by increased progesterone production and expression of inhibin, P450arom, P450scc and LH receptor mRNA. Therefore, with some caution in the interpretation of results, the use of this model appears to be justified for investigating the influence of specific factors on this process.

2 Steroid Action in the Ovary

Prior to the studies described in this thesis, few comparative studies on the effects of androgens and oestrogens on granulosa cells had been carried out. In those which had, the results implied that these classes of sex steroid have very similar effects, with androgens being the more potent, suggesting a similar mechanism of action (Armstrong & Dorrington, 1976; Hillier *et al.* 1985; Hudson *et al.* 1987; Wang & Leung, 1987a; Armstrong & Dorrington, 1976). Indeed, this does appear to be the case for many of the end-points studied in these experiments, such as the effects on patterns of protein synthesis and progesterone production (see Chapters 2 and 3). However, some results described in this thesis suggest that there are differences between the actions of androgens and oestrogens on granulosa cells.

The demonstration of expression of androgen receptor mRNA in rat granulosa cells provided strong evidence that the observed effects of androgens are mediated by the conventional receptor. Expression of androgen receptor mRNA did not appear to be greatly affected by gonadotrophin treatment, suggesting that androgen responsiveness may be independent of the stage of differentiation of the cells. Authentic oestrogen receptor mRNA was detected in RNA extracted from whole rat ovaries by Northern blotting, but none was found in granulosa cell RNA. This does not rule out the possibility that this message is expressed in

granulosa cells, but if so, it is at a very low level. The fact that the oestrogen receptor probe used was transcribed from the mouse cDNA, whereas a rat cDNA was used to detect androgen receptor mRNA, is not likely to account for the failure to detect oestrogen receptor mRNA in rat granulosa cells, since this probe did hybridise to an mRNA of the expected size in rat uterus and ovary RNA samples, albeit less strongly than in mouse tissue. Also, a previous study also failed to detect oestrogen receptor mRNA in mouse granulosa cells using the same probe (Hillier *et al.* 1989a). Since granulosa cells respond so strongly to oestrogen, and since specific nuclear uptake of oestrogen by granulosa cells has been demonstrated (Kudolo *et al.* 1984b; Wolfson *et al.* 1990), it is highly probable that granulosa cells contain conventional oestrogen receptors. Based on these observations, the difference in potency between androgens and oestrogens could be explained in terms of receptor numbers.

If granulosa cells do not express conventional oestrogen receptors, it is difficult to explain the evident effects of oestrogens in these cells. It is unlikely that oestrogens act by binding to the androgen receptor, since it has very low affinity for oestrogens (Schreiber *et al.* 1976; Trapman *et al.* 1988; Lubahn *et al.* 1988), and also because antiandrogens do not antagonise the effects of oestrogens in granulosa cells (although they do block the action of catecholoestrogens) (Hudson & Hillier, 1985; Hudson *et al.* 1987; Spicer & Hammond, 1988b). Mechanisms of steroid action have been proposed which do not involve binding to conventional nuclear receptors. Binding of steroids, and other factors which normally are thought to act via nuclear receptors, to membrane components has been demonstrated, resulting in signal transduction through an intracellular second messenger (Bomsel-Helmreich *et al.* 1979; Farach-Carson *et al.* 1991). This mechanism has not yet been studied in great detail, and current concepts still favour binding of steroids to nuclear receptors, leading to transcriptional activation (O'Malley & Tsai, 1992). The oestrogen receptor probe did hybridise to a smaller (~1.5kb) transcript present in rat granulosa cells, and so it is possible (although not very likely) that this oestrogen receptor-related transcript may be involved in oestrogen action via a mechanism distinct from the conventional

receptor-mediated process. However, it is more likely that this molecule is an intracellular oestrogen-binding protein, which may act to protect the granulosa cell from chronic oestrogen stimulation when the cell is steroidogenically active (Hillier *et al.* 1989a). Certainly, the expression of this message is highest after FSH treatment, when oestrogen synthesis would be at its highest, and declines after hCG treatment following FSH treatment, when oestrogen synthesis would also decrease. If this molecule is involved in oestrogen action, one would expect this to result in increased oestrogen responsiveness with increased maturity. Conversely, if it is a binding protein, one would expect oestrogen responsiveness to decrease with increasing maturity. Therefore, some circumstantial evidence for the function of this factor could be obtained by investigating the relative oestrogen sensitivities of granulosa cells at different stages of differentiation. The true function of the protein encoded by this RNA will probably not be determined until its cDNA is cloned and sequenced.

The significance of the greater potency of androgen compared to oestrogen is not known, but intuitively it seems probable that, since androgen has to diffuse from the theca layer and across the basement membrane before reaching the granulosa cells, its concentration upon reaching those cells will be far lower than that of oestrogen. Oestrogen, on the other hand, can act within the cell in which it is synthesised. Moreover, after being taken up by steroidogenically active granulosa cells, much of the androgen will be rapidly aromatised to oestrogen in the cytoplasm before it can reach the nucleus. Therefore, greater sensitivity to androgen than to oestrogen would seem to be necessary for any response to androgen to occur on one hand, and to prevent hyperstimulation by locally high concentrations of oestrogen on the other. Such a difference in sensitivity to these two classes of steroid would be consistent with a common mode of action for androgens and oestrogens (perhaps by sensitising cells to the FSH-induced elevation of intracellular cAMP levels), with androgens being the more potent simply because they have to be in order to exert the same effect. This implies that the importance of androgen may not be as great as its potency may predict.

Since androgens have been shown to be more potent than oestrogens in augmenting the action of FSH on end-points such as production of progesterone and oestradiol, it was somewhat surprising to find that the opposite may be the case for inhibin gene expression. Where a marked augmentation of inhibin gene expression by steroids was observed, oestradiol appeared to be more potent in this respect than DHT. Moreover, whereas no effect of androgen was observed in the absence of FSH, oestradiol alone consistently stimulated the expression of inhibin-α and -β_B mRNA. These results strongly imply some difference between the modes of action of androgens and oestrogens.

It is not known at what level or levels steroids modulate the transduction of gonadotrophic signals, or whether they have effects which are independent of cAMP. Greater or more prolonged increases in cAMP levels have been shown in granulosa cells stimulated by FSH in the presence of oestrogen or androgen (Goff *et al.* 1979; Hillier & deZwart, 1982; Knecht *et al.* 1985; Fitzpatrick & Richards, 1991), although not all studies have shown any effect on adenylate cyclase or phosphodiesterases (Nimrod, 1977b). Steroids are also capable of augmenting the action of cAMP analogues on granulosa cells (Hillier & deZwart, 1982; Nimrod, 1977b; Knecht *et al.* 1984; Knecht *et al.* 1985), suggesting that, at least in part, their action is exerted at a site distal to the generation of cAMP. A number of intracellular events are involved in the cAMP response between the binding of hormone to its membrane receptor and changes in gene expression and protein phosphorylation events (Montminy *et al.* 1990). Assuming that conventional receptors for androgens and oestrogens are present in granulosa cells, which are considered to exert their effects primarily at the level of transcription (Beato, 1989; O'Malley & Tsai, 1992), it is not inconceivable that the two receptors exert the same effects on the expression of some genes, but different effects on others. According to this hypothesis, where the effects of androgens and oestrogens on cAMP-induced events are qualitatively the same (e.g. steroid production), one would be led to conclude that they affect the same genes (encoding components of the cAMP-response system, perhaps), and differences in potency could be explained in terms of relative receptor numbers. Where there is a basic difference between the

effects of these factors (e.g. inhibin-α and -β_B gene expression), one would conclude that the target genes for the two receptors were different. Until the specific genes in question (which may encode generic components of signal transduction pathways, rather than cell-specific factors such as inhibin subunits or steroidogenic enzymes) are identified and their regulatory regions inspected for steroid response elements, this hypothesis will remain speculative.

3 Inhibin and Activin in Granulosa Cell Differentiation

Before the studies described here were carried out, it was already known that FSH stimulates production of inhibin (Bicsak *et al.* 1986; Suzuki *et al.* 1987; Bicsak *et al.* 1988; Hillier *et al.* 1989b) and expression of inhibin α-subunit mRNA (Woodruff *et al.* 1987) by rat granulosa cells, and *in situ* hybridisation studies had established that the expression of α- and β-subunit proteins and their mRNA increases with the size of the follicle (Meunier *et al.* 1988a; Woodruff *et al.* 1988). After the LH surge, the expression of these genes falls in the ovulatory follicles (which are the only ones which would be expected to contain LH-responsive granulosa cells), with some residual expression of the α-subunit in the newly formed corpus luteum (Cuevas *et al.* 1987; Meunier *et al.* 1988a). The results of the experiments described in Chapter 4 confirmed that the expression of the α-subunit is stimulated by FSH, and also showed, as one would expect, that expression of the other two subunits is also stimulated by FSH. Consistent with the fall in inhibin subunit gene expression in the granulosa cells of large follicles after the LH surge, they also showed that administration of hCG to animals which had been treated with FSH caused a marked fall in levels of inhibin subunit mRNA. All three inhibin subunits seemed to be regulated by gonadotrophins in qualitatively the same way. The expression of P450arom and the LH receptor, and also the oestrogen receptor-related transcript all followed roughly the same pattern, as one might expect if the effects of gonadotrophins on granulosa cell function are mediated primarily through elevation of intracellular cAMP levels. It was not shown whether cAMP was responsible for the fall in LH receptor mRNA in

granulosa cells following hCG treatment, since ligand-induced down-regulation could account for these results (as it probably did in residual ovarian tissue containing theca cells). On the other hand, where hCG was found to affect LH receptor expression in granulosa cells which had not been treated with FSH, it was stimulatory rather than inhibitory. Also, as discussed above, the effects of gonadotrophins on the expression of the oestrogen receptor-related transcript could be indirect, being a response to changing levels of oestrogen within the cell. Despite a common second messenger for gonadotrophins, there are qualitative and quantitative differences not only in the effects of the two hormones, but also in their effects on the expression of different genes. The clearest example of such a difference is the expression of P450scc mRNA, which was stimulated to only a modest degree by FSH, and much more strongly stimulated by hCG in FSH-treated cells, rather than being suppressed, as the expression of the inhibin subunits, P450arom and LH receptor mRNAs were. A less obvious, but certainly distinct difference between regulation by gonadotrophins of the expression of the different genes studied was the effect of hCG administered *in vivo* on granulosa cells which had not been treated with FSH. This treatment was only found to increase the levels of P450scc, P450arom and LH receptor mRNA on one occasion and the effect was very slight, especially on P450scc mRNA levels. However, hCG administered *in vivo* consistently stimulated expression of mRNA encoding all three inhibin subunits in granulosa cells which had not been treated with FSH, in one experiment to an equal or greater degree than FSH (although the dose of hCG was very high in order to mimic an LH surge). This effect was not seen *in vitro*, which may indicate that this effect was due to a thecal product whose production was stimulated by hCG (other possible reasons for this are discussed above). Also, expression of mRNA encoding all three subunits was detectable in freshly isolated granulosa cells from control animals, especially the α-subunit mRNA, which was present at such high levels in untreated cells that gonadotrophins had only slight effects on its abundance. In contrast, no mRNA encoding P450arom, P450scc or LH receptor was detected in cells from control animals. This is consistent with immunocytochemical and *in situ* hybridisation studies in the rat which

have shown widespread expression of α-subunit mRNA even in preantral follicles (Meunier *et al.* 1988a; Meunier *et al.* 1989; Dykema *et al.* 1991), whereas expression of P450scc (Rodgers *et al.* 1986a; Zlotkin *et al.* 1986; Le Goascogne *et al.* 1989), P450arom (Milwidsky *et al.* 1980; Ishimura *et al.* 1989; Sasano *et al.* 1989b) and LH receptor (Oxberry & Greenwald, 1982; Richards *et al.* 1976; Peng *et al.* 1991) have only been found in large antral follicles. Circulating levels of inhibin in the rat increase gradually from early in the follicular phase (Watanabe *et al.* 1990; Hasegawa *et al.* 1989; Fujii *et al.* 1983), whereas oestradiol levels are low until soon before ovulation, at which point they rise sharply (Brandt *et al.* 1990; Dupon & Kim, 1973). These results suggest that expression of the inhibin subunit genes is associated with an earlier stage of follicle development than the other ones studied, and may be due to greater sensitivity to gonadotrophin, or that their expression is under the control of different factors.

An attractive hypothesis has recently been put forward to explain the different effects of FSH and LH despite signal transduction via the same second messenger. It has been shown that FSH is capable of stimulating oestrogen synthesis maximally and progesterone synthesis moderately in cultured human granulosa cells from large follicles, while permitting proliferation to occur, whereas LH maximally stimulates synthesis of both oestrogen and progesterone, and inhibits proliferation (Yong *et al.* 1991b). These effects of FSH could be mimicked by low doses of dibutyryl cAMP, and those of LH by high doses (Yong *et al.* 1991a), suggesting that the different effects of FSH and LH are due to different capacities for generating intracellular cAMP concentrations. The authors suggested that there are fewer receptors for FSH than for LH on mature granulosa cells, resulting in a greater increase in intracellular cAMP levels in response to LH. This theory also explains how low concentrations of LH can mimic the effects of FSH on immature granulosa cells, since those cells would be expected to have fewer LH receptors. These observations are consistent with the proposal that different gonadotrophin-responsive genes have different cAMP dose-response characteristics (Richards *et al.* 1987; Richards & Hedin, 1988). For example, in rat granulosa cells, the expression of P450arom in response to

cAMP would be expected to be biphasic, with stimulation at low doses, and inhibition at high doses, whereas P450scc expression would be expected to be less sensitive to cAMP, and not to be inhibited at high doses. However, it is not known whether this means that gonadotrophin-responsive genes fall into two categories (low and high cAMP threshold), or whether these different genes all have different sensitivities to cAMP. Since expression of the inhibin subunit genes appears to be more sensitive to gonadotrophins than that of the other markers of granulosa cell differentiation studied (see above), it seems probable that the latter is the case. This would imply that the process of granulosa cell differentiation is a gradual one, with expression of genes being sequentially induced as the follicle develops. In situ hybridisation studies have shown that granulosa cell LH receptor mRNA is predominantly expressed in those cells closest to the basement membrane (Camp *et al.* 1991; Peng *et al.* 1991), whereas inhibin subunit mRNA is expressed in all granulosa cells, including the cumulus cells (Meunier *et al.* 1988a; Rivier *et al.* 1989; Dykema *et al.* 1991). Since those granulosa cells closest to the basement membrane are thought to be the most highly differentiated (Zoller & Weisz, 1978; Zoller & Weisz, 1980; Hsueh *et al.* 1984), this pattern of expression is consistent with inhibin expression being associated with an earlier stage of differentiation than other markers such as the LH receptor.

Since inhibin and activin have opposite functions one would expect the control of synthesis of the α- and β-subunits to differ, so it was somewhat surprising to find that expression of the α- and β-subunit genes is regulated in the same way by gonadotrophins. The only difference in the regulation of expression of these genes was the stimulation of expression of α- and β_B-subunit mRNAs by oestradiol, but nor would this provide a mechanism for the preferential production of activin. Because inhibin α-subunit mRNA appears to be more abundant and widely expressed in the ovary than those of the β-subunits (Meunier *et al.* 1988b; LaPolt *et al.* 1989; Penschow *et al.* 1990), one might be led to assume that when β-subunits are produced, the secreted product would be inhibin rather than activin, due to the excess of α-subunits. Of course, the levels of mRNA and its protein product are not necessarily directly

related, since control of production of a protein can be exerted at several levels, so caution must be exercised in extrapolating from mRNA levels to protein production. If activin is produced by granulosa cells in any significant amounts, the control of its synthesis must be at a level other than gene expression, or else its control at that level must be extremely subtle. It is possible that the stimulation of α- and β_B-subunit mRNA by oestradiol is part of a mechanism regulating the different forms of inhibin or activin produced under different conditions, but this result must be interpreted with caution since it was not conclusively shown that the expression of β_A-subunit mRNA is not affected by oestradiol. The most likely explanation for stimulation of inhibin gene expression by oestradiol alone, and for its greater potency than androgen in augmenting the effects of FSH or cAMP, is that inhibin (which stimulates thecal androgen production (Hsueh *et al.* 1987; Hillier *et al.* 1991b)) must be produced in tandem with oestradiol (which inhibits androgen production (Leung & Armstrong, 1979; Magoffin & Erickson, 1982)) to ensure a continued supply of substrate for oestrogen synthesis. Since unstimulated cells contain very low levels of cAMP (Richards *et al.* 1979; Hillier & deZwart, 1982), and since inhibin gene expression is under the control of factors operating through mechanisms distinct from the cAMP pathway (LaPolt *et al.* 1989), it is quite plausible that this action of oestrogen is independent of the cAMP signalling system. Control of inhibin gene expression by multiple signalling pathways could account for the difference between it and those other markers of differentiated function studied, and would suggest that inhibin and activin are produced and act under different conditions from those other factors.

Control of inhibin expression by an "antisense" inhibin α-subunit transcript would add another level of complexity to this subject. However, the nature of the RNA species detected using the inhibin-α sense probe is unknown, and expression of "antisense" β-subunit transcripts was not investigated, so the detection of this molecule must be considered a preliminary finding.

4 Future Prospects

Although the studies described in Chapter 4 showed beyond doubt that the expression of the mRNA encoding all three inhibin subunits is stimulated by FSH both *in vivo* and *in vitro*, the effects of sex steroids and gonadotrophins must be studied in greater detail before the regulation of expression of these genes is fully understood. It would appear from the results that steroids do augment the action of FSH on inhibin subunit gene expression, and that hCG may be stimulatory in immature cells and inhibitory in mature cells, but the effects were variable and were not quantified. Detailed studies of the dose-response characteristics of the expression of these genes in response to gonadotrophins, cAMP and steroids at different stages of maturity would undoubtedly resolve the regulation of the control of these messages, and would also establish the relative roles of androgens and oestrogens in the control of granulosa cell function at different stages of follicle development. In order that meaningful conclusions may be drawn from such data about the role of inhibin and steroids in granulosa cell differentiation, and the sensitivities of different genes to hormonal signals, the expression of the inhibin subunits should be compared to that of other genes under the same conditions. Since Northern blotting is not a quantitative technique, and requires large amounts of RNA for analysis, no attempt was made to use this technique for such studies. Instead, an RNase protection assay was developed for use in these experiments, so that quantitative results could be obtained using a minimum of material. This approach could also have yielded information not available by Northern blotting, such as the direct measurement of the absolute levels of mRNA encoding each inhibin subunit, the detection of any effect of androgen alone on any inhibin subunit mRNA, and the detection of any effect of oestrogen alone on the expression of β_A-subunit mRNA. With this information, conclusions about the production of different forms of inhibin and activin could be drawn. Unfortunately, due to constraints of time available for the completion of this project, the assay was never fully developed and validated, and so it was not possible to carry out any of these studies.

Expression of androgen receptor mRNA was detected in granulosa cells, which did not appear to be regulated by gonadotrophins. However, the control of expression of this message would have to be studied in greater detail before its implications for granulosa cell androgen responsiveness could be inferred. In contrast, no expression of oestrogen receptor mRNA was found. However, it could not be ruled out that this was due to the sensitivity of the method employed in its detection. If these cells do not possess conventional oestrogen receptors, the mechanism of the undoubted effects of oestrogens is a mystery. Therefore, it would be worthwhile to use a more sensitive assay such as RNase protection assay, or even reverse transcriptase-PCR, to establish conclusively whether granulosa cells express this message. Also in relation to oestrogen action in granulosa cells, it would also be interesting to find out what protein (if any) is encoded by the oestrogen receptor-related transcript, and what the significance of its hormonal regulation might be.

Two other observations of unknown significance were made in this project. Firstly, the synthesis of a prominent ~24kDa secreted granulosa cell protein is increased after exposure of cells to FSH. Because this protein is so abundant, and the effect of FSH was seen both *in vivo* and *in vitro*, it seems likely that this protein is involved in the process of granulosa cell differentiation. Therefore, another interesting extension of the work presented in this thesis would be the identification of this protein. Secondly, the detection of a putative "antisense" inhibin α-subunit RNA, and the control of its expression by hCG, has implications for the control by gonadotrophins of inhibin production in granulosa cells. It would be very interesting to characterise this transcript, and to determine its role in the control of inhibin production.

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Appendix Publications

The data published in the following paper are described in Chapter 4 (presented in a slightly different form in Fig.4.11). This work was also presented at the 180th meeting of the Society for Endocrinology, London, November 1989 (*J. Endocrinol.* **123** Suppl., abstract 90).

The data presented in Fig.3.4 was presented at the Society for the Study of Fertility Annual Conference, Sheffield, July 1990 (*J. Reprod. Fertil.* Abstract Series No.5, Abstract 97).

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Regulation of Inhibin Subunit Gene Expression by FSH and Estradiol in Cultured Rat Granulosa Cells.

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Abstract. Roles of follicle-stimulating hormone (FSH) and sex steroids in regulating the expression of mRNA species encoding the α-, β_A- and β_B-subunits of inhibin were studied in cultured granulosa cells from immature rat ovaries. Inhibin subunit mRNAs were detected by Northern blot analysis of total RNA extracted from granulosa cell monolayers which had been incubated for 48 h in serum-free medium containing FSH (100 ng/ml) and/or a steroid (10^{-6} M): estradiol (E), testosterone (T) or 5α-dihydrotestosterone (DHT). Levels of mRNA encoding each inhibin subunit in untreated (control) cultures were low. In cultures treated with FSH alone, levels of inhibin α-, β_A- and β_B-subunit mRNA were approximately 60-fold, 70-fold and 66-fold greater than control, respectively. In cultures treated with E alone, levels of inhibin α- and β_B-subunit mRNA were elevated approximately 4-fold and 2-fold, respectively, but the level of inhibin β_A-subunit mRNA was not measurably affected. Treatment with T or DHT alone had no consistent effect on the levels of any inhibin subunit mRNA. The stimulatory effects of FSH were not consistently altered by the presence of either androgen or estrogen. These results confirm the role of FSH in regulating inhibin α-subunit gene expression and provide direct evidence that both inhibin β-subunit genes are inducible by FSH in granulosa cells. All three inhibin subunit mRNAs followed the same pattern, suggesting that their expression is coordinately regulated by FSH during granulosa cell differentiation.

Introduction

Two distinct forms of inhibin composed of a common α-subunit and one of two β-subunits, β_A and β_B, have been isolated from ovarian follicular fluid (1). The three inhibin subunits are encoded by separate genes whose expression in granulosa cells is developmentally regulated, and therefore likely to be under gonadotropic control (2, 3). Direct evidence exists that expression of the inhibin α-subunit gene is controlled by FSH (4) but there are no equivalent data regarding the control of β_A- and β_B-subunit gene expression. Here, we present direct evidence for coordinate regulation of inhibin α-, β_A- and β_B-subunit mRNA expression by FSH in primary rat granulosa cell cultures. We also show that the α- and β_B-subunit genes are inducible by estradiol.

Materials and Methods

Female 21-day old Sprague-Dawley rats received a 10-mm silastic capsule containing diethylstilbestrol placed subcutaneously to stimulate granulosa cell proliferation. Five days later they were sacrificed by cervical dislocation, the ovaries removed, and granulosa cells from preantral-early antral follicles were harvested in culture medium. The medium was Medium 199 containing 25 mM HEPES buffer, extra (2 mM) L-glutamine, penicillin (50 IU/ml) and streptomycin (50 mg/ml) (all from Gibco Ltd, Paisley, UK) with 0.1% (wt/vol) bovine serum albumin (BSA; ICN Biomedicals, High Wycombe, Bucks, UK). Multiwell plastic dishes (Linbro Space Savers, Flow Laboratories, Rickmansworth, Herts, UK) were precoated with donor calf serum (Gibco) and washed with Dulbecco's phosphate buffered saline before use (5). The culture wells were inoculated with replicate 250 μl portions of cell suspension containing approximately $2x10^5$ live granulosa cells (viability >40% determined by trypan blue exclusion). Human FSH (LER/8-116, 100 ng/ml; ref 6) and/or 10^{-6} M steroid (testosterone [T], 5α-dihydrotestosterone [DHT] or estradiol [E], all from Sigma Chemical Co, Poole, Dorset, UK) were previously added to give a final incubation volume of 500 μl. The cultures were incubated at 37 C in a humidified incubator gassed with 95% air/5% CO_2. After 48 h, the medium was removed and the culture dishes were placed on ice. The cell monolayers were lysed with a solution (300 μl) containing 4 M guanidium thiocyanate, 24 mM sodium citrate, 0.5% (wt/vol) sarcosyl and 0.1 M β-mercaptoethanol. Lysates were pooled from all wells receiving the same treatment (n=8-16) and total RNA was extracted with phenol-chloroform (7). 5μg of total RNA from cells receiving each treatment were separated by electrophoresis in 1.5% agarose gels containing 2.2 M formaldehyde. RNA molecular weight standards (Gibco) were run alongside samples on each gel and ethidium bromide staining was used to check the uniformity of sample loading (Fig. 1). RNA was transferred onto nylon membranes (Hybond-N, Amersham International plc, Aylesbury, Bucks, UK) in 20 x SSC, by capillary blotting. cDNA probes to the α-, β_A- and β_B-subunits of rat inhibin (clones α7, β_A30 and β_B11, ref 8) were labelled with ^{32}P by random priming (Multiprime kit, Amersham). Prehybridization, hybridization and washing were all carried out at 65 C. Blots were prehybridized for 1-2 h in 0.2 M sodium phosphate (pH 7.2), 1 mM EDTA, 1%

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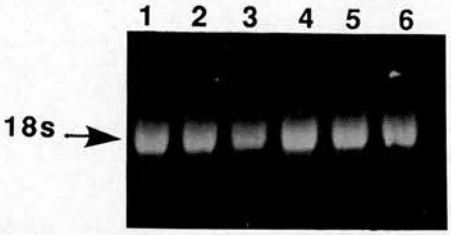

Fig.1. Ethidium bromide stained 18s ribosomal RNA bands, demonstrating uniform gel loading with total RNA. The same gel was used to generate the Northern blots shown in Fig. 2

wt/vol) BSA, 7% (wt/vol) sodium dodecyl sulphate (SDS) and 15% (vol/vol) formamide, then hybridized overnight n the same buffer containing approximately 5 x 10^5 cpm/ml of probe. The membranes were then washed for at least 1h in 40 mM sodium phosphate (pH 7.2), 1 mM EDTA and 1% (wt/vol) SDS with several changes of washing solution, and exposed to Kodak XAR-5 film for -10 days at -70 C using an intensifying screen. Autoradiographic signals were quantified by scanning densitometry.

Results

Northern blots from a representative experiment carried out on seven separate occasions are shown in Fig. 2. Levels of the mRNA species encoding each inhibin subunit were low or undetectable in untreated control cultures. Four inhibin subunit mRNA transcripts were readily detected in FSH-treated cultures: one α-subunit mRNA (~1.7 kb), one β_A-subunit mRNA (~6.5 kb), and two β_B-subunit mRNA species (~4.4 kb and ~3.3 kb). Of the two β_B-subunit transcripts, the larger was the more abundant, in agreement with previously reported findings (8, 9, 10). Treatment with FSH alone stimulated the α-, β_A- and β_B-subunit mRNA levels on average 60 (range 5-258), 70 (9-242) and 66 (12-187) fold respectively, relative to control. Treatment with E alone increased the α- and β_B-subunit mRNA levels 4 (2-10) and 7 (2-25) fold relative to control but had no discernible effect on the β_A-subunit mRNA level. Treatment with T or DHT alone had no consistent effect on the expression of any subunit mRNA relative to control. When combined with FSH, neither androgen nor estrogen consistently altered the response relative to FSH alone.

Discussion

These results show that expression of inhbin α-, β_A- and β_A-subunit mRNAs in rat granulosa cells is under direct control by FSH. The α-subunit data confirm previous evidence that this mRNA is directly inducible by FSH during granulosa cell differentiation *in vitro* (4) and *in vivo* (11). However, the β-subunit data are novel, showing that both β_A- and β_B-subunit mRNAs are also directly inducible by FSH *in vitro.*

The demonstration that granulosa cell inhibin β_A- and β_B-subunit mRNAs are inducible by FSH points to a major intersexual difference in the hormonal control of gonadal inhibin production. Sertoli cells, the testicular homologs of granulosa cells, also express inhibin α, β_A- and β_B-subunit mRNAs (8, 9, 10, 12). However, whereas both granulosa and Sertoli cell α-subunit mRNA production

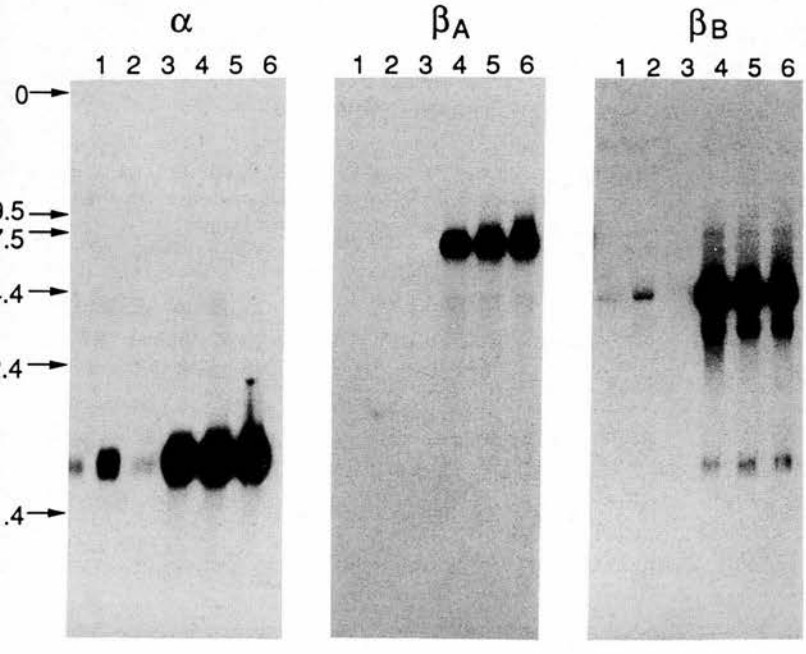

Fig.2. Northern blot analysis of inhibin subunit mRNAs in rat granulosa cells. The cells were cultured for 48 h with or without 100 ng/ml human FSH, in the presence or absence of 10^{-6} M steroid: testosterone (T), 5α-dihydrotestosterone (DHT) or estradiol (E). Total RNA (5 μg) from cells receiving each treatment was separated by electrophoresis on an agarose-formaldehyde gel and transferred to a nylon membrane, which was sequentially hybridised with each ^{32}P-labelled rat inhibin subunit cDNA probe: *left panel*, α-subunit; *centre panel*, β_A-subunit; *right panel*, β_B-subunit.
Key: **1**, control; **2**, E alone ; **3**, DHT alone ; **4**, FSH alone ; **5**, FSH plus E ; **6**, FSH plus DHT.
Numbers on the left denote approximate molecular weights (kb).
The lowest molecular weight species in the right-hand panel is residual inhibin α-subunit probe which was incompletely stripped from the membrane before reprobing.
Exposure times were: *left panel*, 30 h; *centre panel*, 8 d; *right panel*, 10 d. These data are representative of the results obtained from seven individual experiments.

is stimulated directly by FSH, the production of β_A- and β_A-subunit mRNAs by Sertoli cells is unresponsive to FSH *in vivo* and *in vitro* (10, 12, 13). Thus FSH appears to cause differential expression of inhibin subunit mRNAs in Sertoli cells, whereas it effects coordinate induction of inhibin subunit mRNAs in granulosa cells.

Rapid changes in the expression of all three inhibin subunit mRNAs occur in granulosa cells throughout the rat estrous cycle (2). Our results suggest that differential expression of β_A- and β_B- subunit mRNA could involve the action of intrafollicular estrogen. Inhibin production, like other FSH-inducible granulosa cell functions, is augmented by the presence of sex steroids *in vitro* (14, 15), consistent with paracrine regulatory functions for follicular steroids *in vivo* . In a rat granulosa cell culture system similar to that used here, FSH-induced inhibin (measured by RIA directed against the N-terminal sequence of the porcine inhibin α-subunit) was increased by androstenedione and estradiol whereas neither steroid was effective in the absence of FSH (14, 15). Here, we observed direct induction of α- and β_B-subunit mRNAs by estradiol in the absence of FSH, with no consistent interaction between FSH and the estrogen. This could be a mechanism for determining which dimeric forms of inhibin and activin, and which free subunits predominate at different stages of follicular development.

The intraovarian functions of the various forms of inhibin, activin and free subunits present in follicular fluid are still unclear (16, 17, 18). However, this study suggests that the genes which encode all three subunits are under primary endrocrine control by FSH. A secondary paracrine level of control, mediated by estrogen, is also implied. It remains to be seen whether other follicular factors which influence inhibin production (14, 19) do so by affecting inhibin subunit gene expression directly.

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